KINETIC AND CYTOLOGICAL ANALYSES OF PLANT TISSUE CULTURES - AN APPROACH TO GENETIC MANIPULATION OF PLANT SOMATIC CELLS

by

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Declaration

I declare that all work presented in this thesis, is original work, entirely carried out by myself, unless otherwise acknowledged.

Sarah Ashmore

Sarah Ashmore
May, 1981

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ABSTRACT

This thesis presents a study of tissue culture systems of 3 low chromosome number composites, *Crepis capillaris* (2n = 6), *Haplopappus gracilis* (2n = 4), and *Brachycome dichromosomatica* (2n = 4), with a view to their being used in genetic manipulation experiments, including DNA uptake and fusion studies. An initial description has been made of the chromosomal characteristics of cell cultures of these 3 species, which includes the use of the Giemsa C-banding technique. A common pathway of karyotype evolution has been observed in these cultures. In addition, the C-banding analysis of a tumorous suspension cell culture of *Crepis capillaris*, CAPT, has revealed extensive chromosomal rearrangements, such as have not previously been reported in plant tissue cultures. Cell cycle analysis of the CAPT culture, using several different approaches, has uncovered an unusual model of cell kinetics, with a low growth fraction and a very short, or absent, G_1 phase in cycling cells.

Attempts at DNA binding, and the binding of liposomes of 3 different lipid compositions, to protoplasts, as analysed using autoradiography, were successful. A model of variation in surface charge with cell cycle progression has been proposed based on analysis of binding to protoplasts at different stages of the cell cycle. This model has been supported by the use of 2-polymer phase separation of protoplasts.

Fusion of protoplasts was achieved using 3 different chemical fusogens, polyethylene gycol (PEG), liposomes and concanavalin A (Con A). Analysis of binucleates has revealed that cell cycle combinations occur in a close to random fashion, although such deviation from randomness as does occur is in the direction of like/like cell cycle combinations, which perhaps fits with the model of charge variation through the cell cycle which has been proposed. Differences in binucleate analyses for the 3 fusogens, suggesting differences in their modes of action, are discussed.

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GENERAL INTRODUCTION

Cells which normally form part of higher organisms may be removed from their multicellular environment and maintained in a viable, dividing state for prolonged periods in specialised media (Paul, 1960). Such long-term *in vitro* culturing of cells was first demonstrated by Harrison in 1907 using cells from the spinal cord of a frog. Since then tissue culture techniques have improved greatly and extensive use has been made of the large relatively homogeneous cell populations available in tissue cultures, for studies of physiological and developmental processes.

The ability to transfer whole genomes between mammalian cells by cell fusion (both intra and interspecific) using inactivated sendai virus (Harris and Watkins, 1965) has been the cornerstone of somatic cell genetics and gene mapping. The phenomenon of chromosome segregation in hybrid cells, first noticed by Weiss and Green (1967) in human/mouse hybrids has become a powerful tool for the production of genetic maps in eukaryotes (Boone and Ruddle, 1969; Boone *et al.*, 1972), and over three quarters of the genetic loci now described in the human genome have been located by the use of hybrid cells. Cell hybrids involving one malignant parent are valuable for the investigation of the genetic control of malignancy (Defendi *et al.*, 1967), and yield information which may aid in the management of this widespread disease of man (Sidebottom, 1980). Well defined mutants in mammalian cells in culture allow specific isolation of heterokaryons. By using two parental cell lines which carry different markers one can establish a selective system wherein hybrid cells will grow but parental cells will not, as in the HAT hybrid selective system, which was devised by Littlefield in 1964, and is now extensively used in mammalian systems.

Most recent investigations into the genetics of animal cell cultures have centred on the transfer of discrete portions of the genome of one organism into the genome of a different organism. Genetic modification of organisms in this way has enormous potential for contributing to several areas of biology, including the exciting future possibility of the repair of genetic disease in man, or the production of new genetic varieties of livestock by simple gene addition. Such unorthodox manipulations would circumvent the need for the time consuming breeding experiments usually required to generate new phenotypes. Additionally, the use of small fragments of genetic information, and even single genes, allows new insights into fine resolution gene mapping and the control of gene expression, including the genetic control of differentiation and development. Foreign genes have been successfully introduced and expressed in mammalian cells in the form of metaphase chromosomes (McBride and Ozer, 1973; Willecke and Ruddle, 1975), as naked interphase DNA (Wigler *et al.*, 1979a; Willecke *et al.*, 1979) or as single genes which have been cloned into prokaryotic vectors for transfer into host cells (Merril *et al.*, 1971; Wigler *et al.*, 1979b). The sophisticated techniques which allow single gene transfer and gene specific probes for their detection in transformed cells (see Shows and Sakaguchi, 1980), address the question of the nature of gene interaction and expression in mammalian cells at the molecular level.

The use of metaphase chromosomes for the introduction of exogenous genetic information may offer certain advantages in terms of biological fitness since the foreign genes are being introduced in their natural form, as chromatin. Techniques are now being developed to separate individual chromosome types by flow microfluorimetry, and have already yielded individual chromosomes, or groups of chromosomes, in high purity, from several mammalian genomes (Gray *et al.*, 1975; Carrano *et al.*, 1979).

Preliminary studies using several different fluorescent DNA stains (Hoechst 33258, DAPI, chromomycin A_3, ethidium bromide and propidium iodide) have revealed that the relative fluorescence of individual chromosome types varies with the stain used, indicating that individual chromosome types differ in their chemical properties (Langlois *et al.* 1980). Thus, the chromosomes may be separated in terms of size and chemical characteristics.

An additional possibility for genetic manipulation, which has received some attention recently, is the entrapment of foreign genetic material inside lipid vesicles (liposomes) which may then be fused with mammalian cells (Poste, in press). The liposomes may provide some protection for the introduced molecules, and, additionally, appear to have no cytotoxic effect on host cells. Targeting of liposomes through incorporation of antibodies into the lipid bilayer opens up their potential usage for directed drug therapy, or, possibly, gene therapy in specific tissues in man.

Whilst such great advances are being made with mammalian tissue cultures, less progress has been made with plants. It was not until 1934 that White reported the first successful, long term, culturing of plant cells, but by the 1950's it was possible to grow viable, highly separated plant cells in both liquid media and on agar plates. Despite the availability of such cultures, and therefore the potential for studies on somatic cells of plant origin, the field of plant tissue culture lagged far behind that of animal tissue culture. For example, although there have been several reports of chromosome segregation occurring within plant heterokaryons (Power *et al.*, 1975; Gleba and Hoffmann, 1979), no use has been made of these hybrid cells for classical genetic studies. Similarly,

there have been no unambiguous reports of the uptake and expression of foreign genetic material in plant cells. The medical relevance of many of the genetic studies in mammalian cells has, perhaps, motivated accelerated progress in this field as compared with plant tissue culture. The genetic manipulation of plant cells must, however, be equally important in human terms, as a means of producing improved crop plants within species which are sexually incompatible and cannot therefore be produced by normal breeding programs. In a world where increasing demand is being placed on the limited food supplies and energy resources, the improvement of agricultural plants by the introduction of specific genes which, for example, confer on plants the ability to resist certain diseases, or, possibly, the ability to fix nitrogen from the air, would be of vital importance.

Aside from the far-reaching possibilities for the use of plant tissue cultures for agricultural purposes, plant systems offer certain advantages over animal tissue cultures for basic investigations at the cellular or whole plant level. The media which support the growth of plant cells are much less complex, consisting of inorganic salts, trace elements, vitamins, a carbon source, and plant growth regulators. Haploid cultures are readily obtained from many plants by the technique of anther culture (Nitsch and Nitsch, 1969), and these cultures are valuable for genetic studies, particularly for the isolation of recessive mutants. Plant cells, unlike animal cells, may be regenerated into whole organisms in the presence of a particular balance of growth regulators (hormones). This means that genetic manipulations at the cellular level may be evaluated at the whole plant level, and that the detection of mutants which are specifically blocked in developmental processes should be possible. Although the majority

of successes with whole plant regeneration have been with plants which have no agricultural importance, amongst the agricultural Gramineae plants can be regenerated from callus of wheat (Shamida *et al.*, 1969), rice (Nishi *et al.*, 1968), sugar cane (Nickell and Heinz, 1974), maize (Green and Phillips, 1975), barley (Cheng and Smith, 1975), and oats (Lörz *et al.*, 1976). It is even feasible to select for high frequency of regeneration as shown by Bingham *et al.* (1975) in a line of alfalfa, where after two cycles of recurrent selection, the frequency of regenerating genotypes increased from 12% to 67%.

In terms of genetic manipulation of plant cells, the infection mechanism of *Agrobacterium tumefaciens*, which cause crown gall disease in dicotyledonous plants and some monocoltyledonous plants, may provide a natural vehicle for the introduction of foreign genes into plant cells. The Ti plasmid of *A. tumefaciens* enters wounded cells, and a part of the plasmid known as the T-DNA region becomes integrated into the plant genome (Chilton *et al.*, 1977). Thus, it may be possible to introduce foreign genes into plant cells by integrating them into the Ti plasmic, the wide host-range of *A. tumefaciens* making it excellent for such an infection process. Plant tumour cells are characterised by their ability to grow, in tissue culture, in the absence of added phyto-hormones, and often by their capacity for synthesis of one or two unusual types of amino acid derivatives. Such markers should be useful in the selection of transformants or as part of a selective system for the isolation of hybrid cells, and in fact Martón *et al.*, (1979) have already shown the recovery of hormone independent callus from tobacco protoplasts treated with *A. tumefaciens*.

Thus, plant systems may hold answers to problems in areas of development and differentiation which cannot be tackled in animal tissue cultures, and certainly offer great potential for studies of a genetic nature. It is interesting to question why it is that the field of plant cell culture has not advanced to the same extent as animal tissue cultures. One of the major problems in plant systems is that the cell wall must be removed, to produce protoplasts, before either fusion or uptake experiments can be attempted. Although simple reproducible techniques for enzymatic digestion of the cell wall are now available, these methods were not introduced until 1960 (Cocking), and often have to be modified considerably for different species of plants, different cell lines, and even for cell cultures in different phases of growth (Uchimiya and Murashige, 1974). Since plant cells in culture normally grow in clumps this has also presented an obstacle to genetic studies of somatic cells, which requires manipulations at the single cell level. The introduction of protoplast isolation and culturing techniques have now circumvented this problem. The recently developed method of cell fusion using polyethylene glycol (PEG) (Kao and Michayluk, 1974) makes fusion between two cells of widely different types both feasible and simple, and has similarly revolutionised the procedures for animal cell fusion. However, despite the ease of inducing cell fusion, effective hybrid selection systems are sadly lacking in plants, mainly due to the difficulty of isolating stable mutants from plant cells. Part of this difficulty may lie in the apparent redundancy of many of the genes in plant cells, as suggested by the leaky nature of some plant mutants. Even the mutant strains isolated by Carlson (1970) from a haploid line of *Nicotiana tabacum* have proved to be leaky. Some authors have avoided the use of mutants in heterokaryon selection by utilising differential drug

sensitivities (Power *et al.*, 1976), chlorophyll deficient plants (Melchers and Labib, 1974), and, in one case, the naturally occurring tumorous nature of the hybrid plant (Carlson *et al.*, 1972). Nevertheless, the problems of mutant isolation pose a real barrier to genetic studies and probably represent the greatest obstacle to be overcome in the field of plant cell genetics. The sophisticated techniques, used with animal cells, for single gene introduction and subsequent detection in resultant transformants, cannot be developed in plant cells without the availability of well defined genetic mutants.

The use of advanced cytogenetic techniques in plant tissue culture could provide markers in the absence of the more specific markers provided by genetic mutants. By the use of chromosome banding techniques (Hsu, 1973), individual chromosomes, and even specific regions of chromosomes, can easily be identified. This allows more accurate description of chromosomal change in cell cultures, particularly in hybrid cells, and provides more specific information on chromosomal location of genes in mapping procedures. Unfortunately, chromosome banding methods have not been exploited in plant tissue cultures, and this again represents a major barrier to more detailed work with plant cells in culture. The reasons for this particular gap in plant systems presumably relates to the poor choice of species for such work, i.e. species which have large numbers of small chromosomes and are therefore generally unsuitable for cytogenetic analysis (see Gleba and Hoffmann, 1979).

Apart from these technical problems, there has been some controversy regarding claims of transformation in plant cells. For example, Ledoux and co-workers examined the interaction of bacterial DNA with germinating seedlings, and claimed that the foreign genetic material had become

covalently bound to the plant genome (see Ledoux, 1974). Ledoux's evidence for such a conclusion was based on the presence of a replicating DNA moledule in the seedlings which has a bouyant density in CsCl intermediate in value to the whole plant DNA and the donor bacterial DNA. This unusual DNA molecule was not observed by Kleinhofs *et al.* (1975) in a similar experiment, and they concluded that a replicating DNA molecule of a bacterial contaminant of the original seed was probably responsible for Ledoux's findings. A number of other studies in this field have been ambiguous, and authors have often drawn firm conclusions from equivocal data. This, at one time, led to considerable confusion and dissillusionment amongst workers involved in the unorthodox genetic manipulation of plants. Indeed the field itself suffered from suspicions generated by some of the early work.

Part of the problem has been poor choice of experimental material, as, perhaps, with Ledoux's work, but also the approaches to plant transformation studies have often been geared to obtaining rapid, sensational results, with no attempt at systematic characterisation of the cell systems being used. The interaction between the plant cell membrane and, for example, DNA, may be affected by pH changes, cycle phase of the cells (which is known to alter membrane physiology), osmotic state of the cells, and other such conditions which can easily be monitored. It may be that the plant cells exhibit a state of competance for transformation at a particular stage of the cell division cycle, as with bacterial cells (Hotchkiss, 1954). Additionally, it should be possible to follow the movement of the introduced material by cytological investigation, including autoradiography. Such techniques may produce the hard quantitative data required in this field of study.

If progress in somatic cell genetics of plants is to be accelerated,

there are then certain central problems which should be tackled. One of these problems is choice of experimental material. Although much effort has been directed towards the development of "model vectors" for genetic manipulation experiments in plants (e.g. Schilperoort *et al.*, 1978), not much attention has been focused on the choice of model hosts for such investigations. In this project, three low chromosome number composites have been used in studies of DNA binding, protoplast fusion, and preliminary observations on the use of liposomes as possible vehicles for the introduction of foreign genetic material into plant cells. The three species, *Crepis capillaris* (2n = 6), *Haplopappus gracilis* (2n = 4), and *Brachycome dichromosomatica* (2n = 4), have been chosen for this study, to evaluate their potential for use as model hosts for unorthodox genetic experimentation. Certainly, the low chromosome numbers and distinctive morphological characteristics of the chromosomes in all three species allow cytological investigation at the single chromosome level, which has been difficult in many of the plants previously utilised for fusion and uptake studies. This probably represents the biggest advantage gained in using these three members of the Compositae. Although this work does not include the isolation of mutants, use has been made of cytogenetic analysis, including chromosome banding, which should at least provide some markers in the karyotype which may be detected in genetic manipulation experiments. It is hoped that future work with these plants will include attempts at isolation of genetic mutants since the lack of specific genetic markers represents such an obstacle to progress in plant somatic cell studies.

Cell cultures, which are maintained both as callus and in suspension for all three species, provide sterile material for experimentation, thereby avoiding problems of contamination in the original host cells, as

with Ledoux's work. Also, the use of rapidly dividing suspension cell cultures should provide a rich source of readily isolated protoplasts, so that manipulations can be carried out at the single cell level, without the barrier of the plant cell wall. Protoplasts at all stages of the division cycle should be released from these cultures, so that cell cycle related phenomena can be investigated. Competance for transformation may only occur at one discrete part of the cell division cycle. Observations of Farber *et al.* (1975) on mammalian cultures, indicate that cells in exponential growth ingest more DNA than cells in stationary phase. If similar effects occur in tissue culture cells of these plants, it may be possible to increase the efficiency of transformation by selecting as host cells only those cells which are in the optimum "state" for the introduction of foreign genetic information.

These three species are not only available in tissue culture, but it is also possible to regenerate whole plants from callus cultures of *Crepis capillaris* (Sacristán and Wendt-Gallitelli, 1973), although this has not been attempted in this project, and from callus cultures of *Brachycome dichromosomatica* (Gould, 1979a). Thus it should be possible in these plants to study the effects of genetic manipulation in somatic cells or the whole plant. It has also been possible to obtain haploid tissue from anther culture of *B. dichromosomatica* (Gould, 1979b).

A tumour derived culture of *Crepis capillaris* has been used in much of the experimentation to be described. This culture, which is phytohormone independent for growth, may provide a marker for the selection of possible heterokaryons or transformants.

As a prerequisite for carrying out experiments which may change the genotype and the phenotype of cells, it is necessary to describe the

characteristics of the original host cells. Thus, the first two chapters of this thesis are devoted to establishing, firstly, the chromosomal make-up of the three plants and their derived tissue cultures, and, secondly, the details of the cell division process in culture. Chapters three and four concern the binding of DNA and liposomes to protoplasts and the fusion of protoplasts. A quantitative cytological approach has been exploited throughout this fusion and binding work.

GENERAL MATERIALS AND METHODS

0.1 WHOLE PLANT MATERIAL (see Fig. 0.1)

0.1.1 *Crepis capillaris*

Seeds (supplied by the Botanic Gardens, Berlin) were germinated on wet filter paper at 22°C in the light, and plants were maintained in a glasshouse, with a diurnal temperature range of 15°C to 24°C. Flowering occurred on a 16 h day, 8 h night regime.

0.1.2 *Haplopappus gracilis*

Seeds (kindly provided by R.C. Jackson) were germinated on wet filter paper at 22°C, in the light, and plants were then maintained in a glasshouse with a diurnal temperature range of 15°C to 24°C. Flowering occurred on a 10 h day, 14 h night regime. *H. gracilis* is found from Southern Colorado and Utah, to the State of Durango in Mexico, and west to the eastern border of California (Hall, 1928).

0.1.3 *Brachycome dichromosomatica*

Seeds of this plant were collected from Wild Dog Glen in the Flinders Ranges of South Australia (site referred to by Watanabe *et al.*, 1975), in early September 1976 by Gould, and by the present author in the following September. Plants have been maintained in a controlled environment cabinet (day length 18 h, day temperature 24°C, night temperature 18°C) where they produce many flowering scapes, and grow much larger than in the field. The seeds of *B. dichromosomatica* contain strong germination inhibitors which may be overcome by long periods (up to 70 days) of leaching in tap water (see Gould, 1979a).

FIG. 0.1

Crepis capillaris

Haplopappus gracilis

Brachycome dichromosomatica

0.2 CELL CULTURES

The callus and suspension cell cultures derived from these species, and used in the present study, are listed in Table 0.1. Their origin, culture designation, characteristics, and the media on which they have been maintained, are documented in this table. Callus cultures were grown on solid agar media, incubated in the dark at 26°C, and regular transfers to fresh medium were performed every 3 to 4 weeks depending on the growth rate of the particular culture (see Fig. 0.2). Callus cultures which were originally set up at R.S.B.S. were obtained by laying cut surfaces of hypochlorite sterilised tissue onto agar medium, followed by incubation as above. Stock suspension cell cultures (60 ml) in 250 ml Erlenmeyer flasks were grown in the dark at 26°C with rotary shaking (150 rpm with a ½" throw) (see Fig. 0.3). Regular transfers were performed by innoculating 10 ml of 7 day old suspension into 50 ml of fresh medium. The *Nicotiana sylvestris* suspension cell culture is maintained in suspension as above, and has been used in some comparative studies in this project.

0.3 CULTURE MEDIA

Three different media have been used (see Table 0.1). These are Miller's B medium (Miller, 1963), B5 medium (Gamborg and Eveleigh, 1968), and Linsmaier and Skoog's medium. The precise compositions of the media used in this study are given in Tables 0.2 to 0.4.

0.4 VIABILITY ESTIMATION

The viability of both suspension cells and protoplasts was estimated, under the microscope, after a 5 minute treatment in Evans blue stain (0.25

TABLE 0.1 - LIST OF CULTURES USED, AND THEIR CHARACTERISTICS

Species	Culture Designation	Origin	Characteristics	Medium type
Crepis capillaris	CAP	Tübingen (Sacristán)	Callus	B5
	CAPT	" "	Tumour derived callus and suspension, (Sacristán and Melchers, 1970)	B5 without 2, 4-D
Haplopappus gracilis	HA-1	Saskatoon (Gamborg)	Aneuploid suspension (also kept as callus)	B5
	HA-new	RSBS shoot derived.	Callus	B5
	HA-3	" " "	Suspension derived from HA-new.	B5
Brachycome dichromosomatica	LIN 31	RSBS leaf and bud derived.	Callus and suspension, initially embryogenic.	Miller's B, supplemented with α-naphaleneacetic-acid (NAA) at 0.5 mg l^{-1} and kinetin at 0.1 mg l^{-1}. Also, B5.
	LIN 113	RSBS	Callus, embryogenic	" " "
Nicotiana sylvestris	NS-1	Leicester (Dix)	Tetraploid suspension	Linsmaier and Skoog medium (1965)

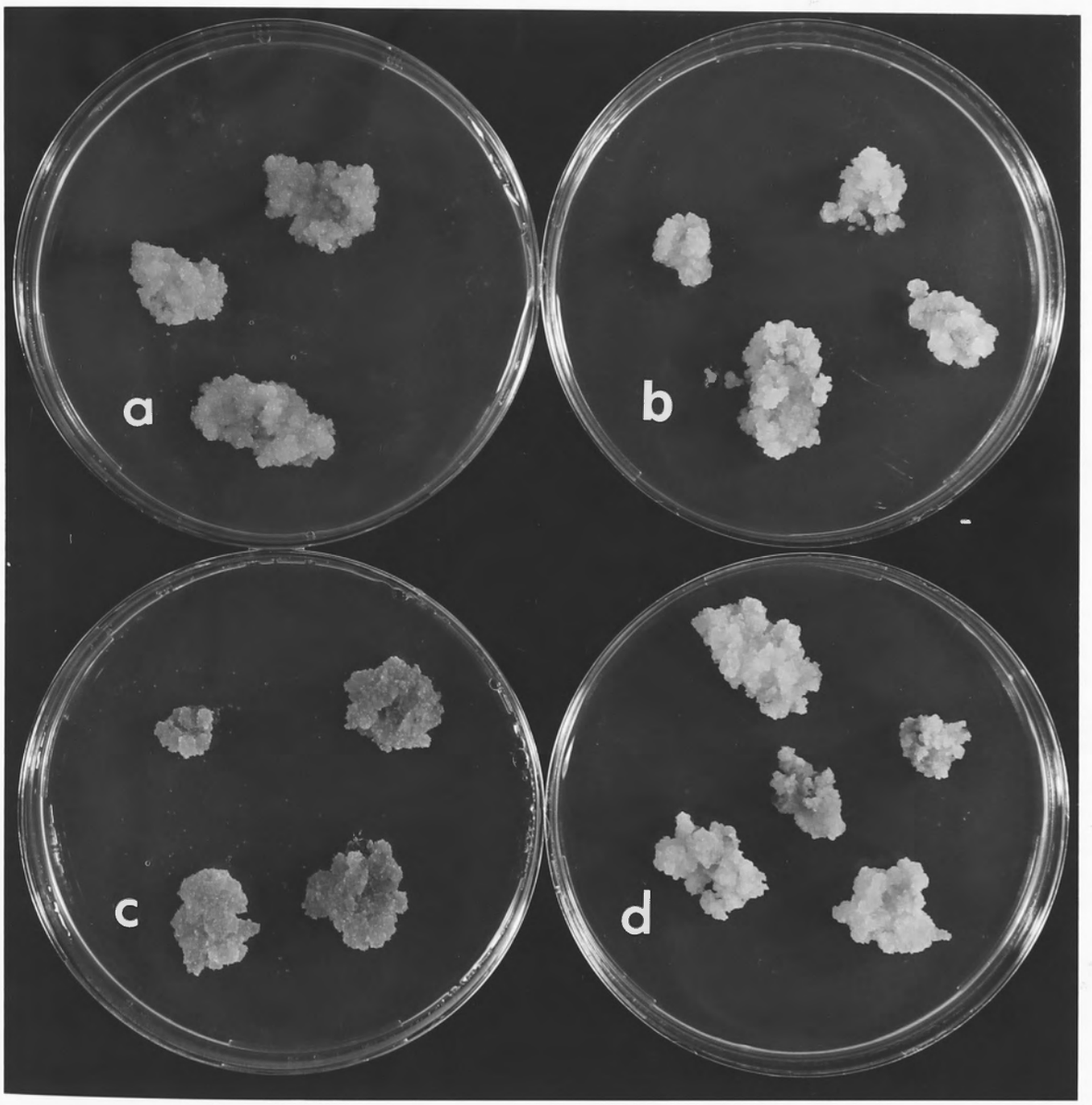


FIG. 0.2 Callus cultures of (a) HA-1; (b) CAPT; (c) Lin_{31}; and (d) NS-1.

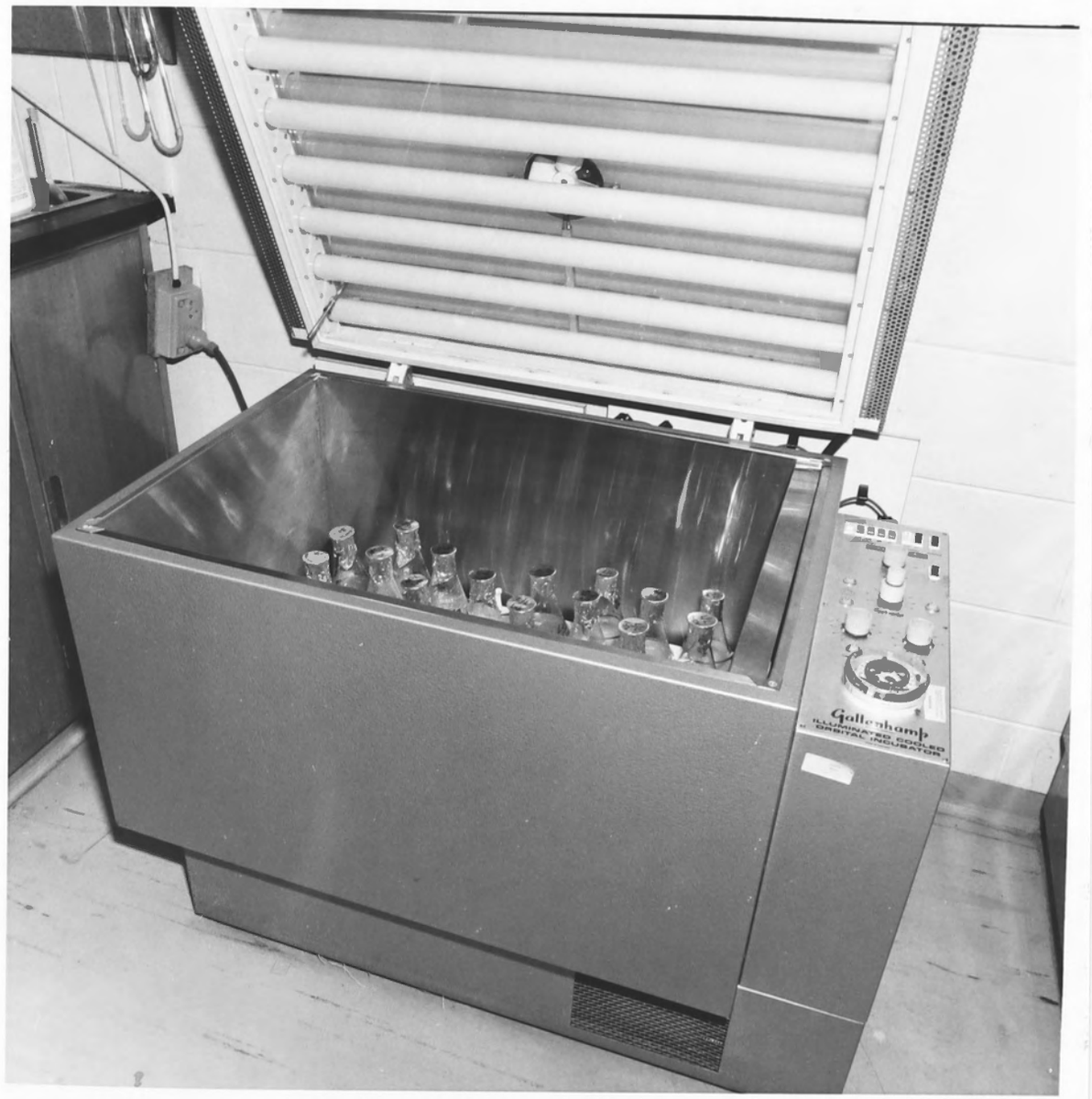


FIG. 0.3 Gallenkamp illuminated cooled orbital incubator used for maintenance of suspension cell cultures. Flasks were incubated in the dark at 26°C with rotary shaking at 150 rpm.

TABLE 0.2 - MILLER's B MEDIUM

Component	Final Concentration (mg/L)	Final Molarity (mM)
Major salts		
NH_4NO_3	1000	12.5
KNO_3	1000	9.9
KH_2PO_4	300	2.2
$Ca(NO_3)_2.4H_2O$	500	2.1
KCl	65	0.87
$MgSO_4.7H_2O$	72	0.3
Minor salts		
$MnSO_4.H_2O$	4.9	0.029
H_3BO_3	1.6	0.026
$ZnSO_4.7H_2O$	2.7	0.0094
KI	0.8	0.0048
Micronutrients		
Nicotinic acid	0.5	0.0041
Pyridoxine-HCl	0.1	0.0005
Thiamine-HCl	0.1	0.0003
Glycine	1.0	0.013
Fe Chelate		
$FeSO_4.7H_2O$	23.95	0.086
Na_2 EDTA	32	0.086
Sucrose	20,000	58.4

pH adjusted to 5.8

TABLE 0.3 - B5 MEDIUM

Component	Final Concentration (mg/L)	Final Molarity (mM)
Major salts		
KNO_3	2500	25
$MgSO_4.7H_2O$	250	1.0
$CaCl_2.2H_2O$	150	1.0
$(NH_4)_2SO_4$	134	1.0
$NaH_2PO_4.2H_2O$	150	0.9
Minor salts		
$MnSO_4.4H_2O$	10	0.04
H_3BO_3	3.0	0.05
$ZnSO_4.7H_2O$	3.0	0.01
KI	0.75	0.005
$Na_2MoO_4.2H_2O$	0.25	0.001
$CuSo_4.5H_2O$	0.25	0.0010
$CoCl_2.6H_2O$	0.25	0.001
Micronutrients		
Nicotinic acid	1.0	0.008
Pyridoxine - HCl	1.0	0.005
Thiamine - HCl	10	0.03
Myo-inositol	100	0.56
Fe Chelate		
$FeSO_4.7H_2O$	27.85	0.1
Na_2-EDTA	37.25	0.1
Sucrose	20,000	58.4
2,4-D	2	0.009
Cas-amino acids	1.0	
pH adjusted to 5.5		

TABLE 0.4 - LINSMAIER AND SKOOG MEDIUM (L AND S)

Component	Final Concentrations (mg/L)	Final Molarity (mM)
Major salts		
NH_3NO_3	1650	20.6
KNO_3	1900	19
$MgSO_4.7H_2O$	370	1.5
$CaCl_2.2H_2O$	440	3.0
KH_2PO_4	170.6	1.25
Minor salts		
$MnSO_4.4H_2O$	22.3	0.1
H_3BO_3	6.2	0.1
$ZnSO_4.7H_2O$	8.6	0.03
KI	0.83	0.005
$Na_2MoO_4.2H_2O$	0.25	0.001
$CuSO_4.5H_2O$	0.025	0.0001
$CoCl_2.6H_2O$	0.025	0.0001
Micronutrients		
Thiamine - HCl	0.04	0.0001
Myso-inositol	100	0.56
EDTA ferric monosodium salt.	37.25	0.1
Sucrose	40,000	117
2,4-D	0.4	0.002
Kinetin	0.03	0.0001
Cas amino acids	1000	

pH adjusted to 5.6

per cent w/v). Live cells retain their semi-permeable properties and therefore exclude Evan's blue whereas dead cells become pigmented as the dye penetrates (Gaff and Okong'O-Ogola, 1971). The validity of this method has been checked by reciprocal staining with fluoroscein diacetate, which causes live cells to fluoresce (Widholm, 1972).

0.5 FEULGEN STAINING OF CELLS AND PROTOPLASTS

Feulgen stain was prepared by overnight decolourisation of a 0.5% solution of basic Fuchsin (Sigma), with 1 M HCl (10 ml to 100 ml solution) and potassium metabisulphite (0.5 g to 100 ml solution). Since decolourisation was often incomplete by the morning, further reduction was performed with activacted charcoal, or by bubbling SO_2 through the solution. Feulgen-DNA contents of carrot root tip meristematic cells, as measured by microspectrophotometry, indicated that the addition of charcoal rather than SO_2 gas provided the most effective stain (Fig. 0.4). In fact, the untreated, pinkish-coloured stain was more potent in its reaction with carrot DNA than the SO_2-treated solution.

Cell Staining

Cells, fixed in 3:1 ethanol:acetic acid at 4°C, for at least 24 h, were washed several times in distilled water, and treated with 1 M HCl at 60°C, for 12 min, prior to staining in Feulgen for 2 hours. A 5 min wash in SO_2 water (freshly prepared 1:1 mixture of 10% potassium metabisulphite and 0.5 M sulphuric acid), was performed to remove all "loosely-bound" Feulgen stain. Cells were then transferred to 45% aqueous acetic acid for softening, before preparation of squashes. Cover slips were removed in dry ice, after the method of Conger and Fairchild (1953) and the slides either stored in water, prior to the preparation of autoradiographs, or

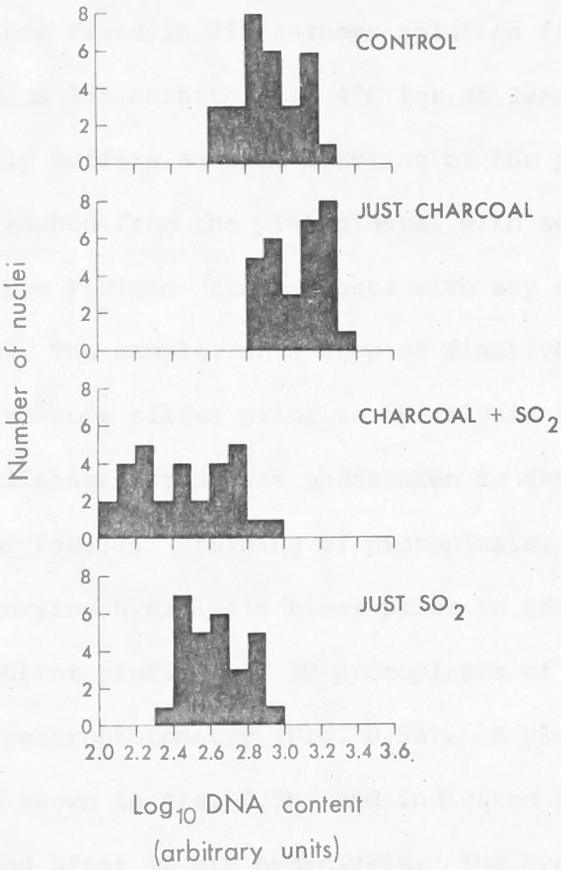


FIG. 0.4 DNA profiles of carrot root tip meristematic cells stained with 4 different Feulgen preparations (a) control (*i.e.* no charcoal or SO_2); (b) stain decolorised using charcoal; (c) stain decolorised using charcoal and SO_2; (d) stain decolorised using just SO_2.

permanently mounted in either Gurr's XAM neutral mounting medium or DEPEX.

Protoplast Staining

Protoplasts were fixed in 75% carnoys solution (3:1 ethanol:acetic acid), in water, plus 13% sorbitol, at 4°C for at least 24 h. The sorbitol osmotically buffers against bursting of the protoplasts, but must be carefully washed from the protoplasts, with several changes of fresh fixative, since Feulgen stain reacts with any sorbitol remaining in the protoplasts. The sample, in a drop of fixative, was then thoroughly air-dried onto microscope slides prior to hydrolysis in 1 M HCl, at 60°C for 12 min. A preliminary study was undertaken to determine the optimum hydrolysis time for Feulgen staining of protoplasts. CAPT protoplasts were treated for varying hydrolysis times prior to addition of stain, and Feulgen DNA-content profiles of 30 protoplasts of each sample were obtained by microspectrophotometry (Fig. 0.5a). A plot of main G_1 values for each sample is shown in Fig. 0.5b, and indicates that the optimum staining is achieved after 12 min hydrolysis. The hydrolysis treatment was followed by Feulgen staining for 2 h, and a 5 min wash in SO_2 water. Slides were then washed in distilled water, and either stored in water prior to the preparation of autoradiography or permanently mounted in either Gurr's XAM neutral mounting medium, or DEPEX.

0.6 AUTORADIOGRAPHY

Subbed microscope slides were used in autoradiograph preparations, to ensure good wet-adhesion of the emulsion during processing. The subbing solution used consists of 1 g each of Kodak purified gelatin, and chrome alum (chromium potassium sulphate) dissolved in 1 litre of

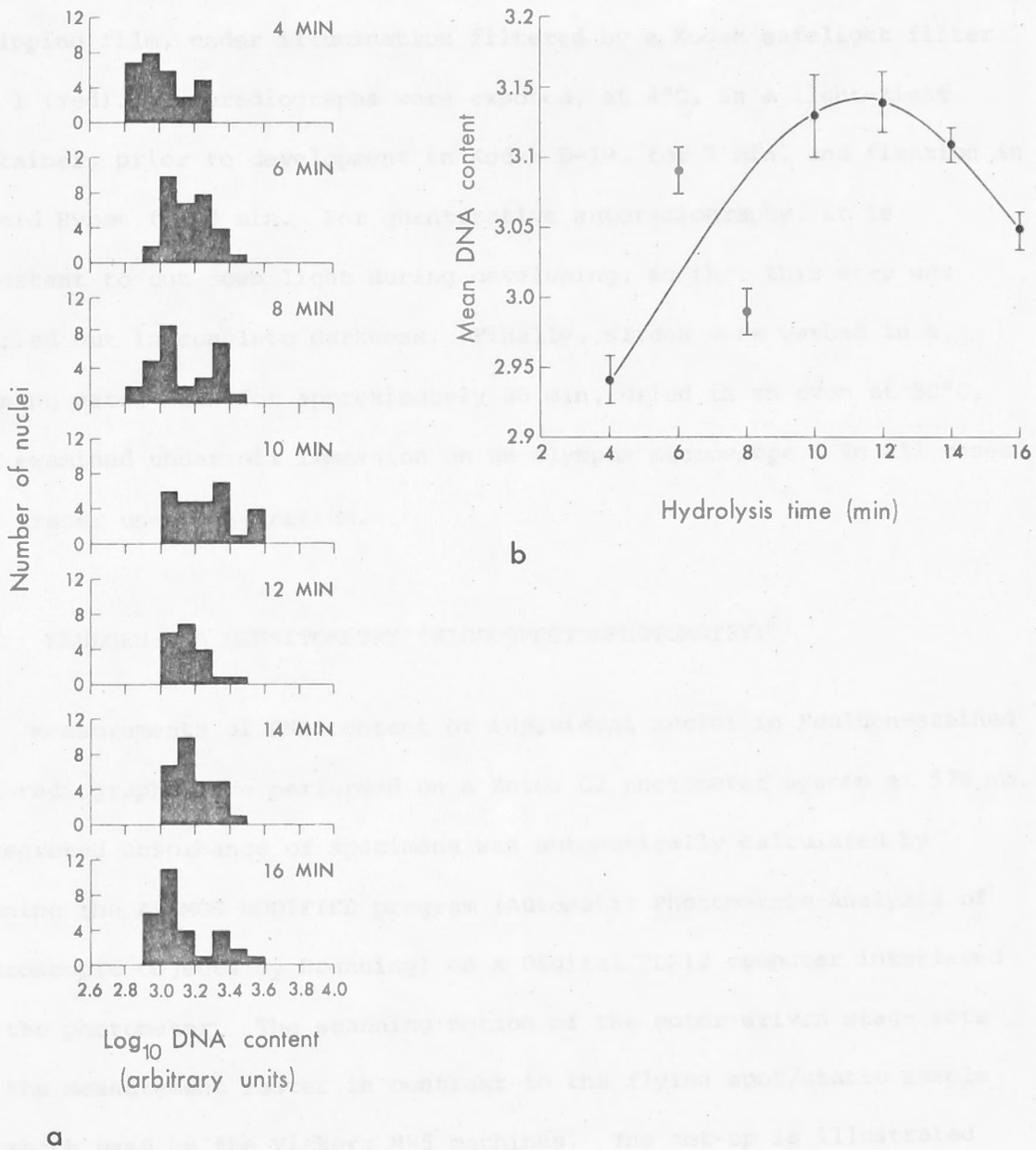


FIG. 0.5 (a) DNA content profiles of CAPT protoplasts treated for varying hydrolysis times prior to addition of Feulgen stain.

(b) Mean Feulgen densities of G_1 nuclei of CAPT protoplasts over a range of hydrolysis times.

water. Prepared slides, previously left soaking in distilled water, were each coated with a layer of Kodak AR.10 fine grain autoradiographic stripping film, under illumination filtered by a Kodak safelight filter No. 1 (red). Autoradiographs were exposed, at 4°C, in a light-tight container, prior to development in Kodak D-19, for 5 min, and fixation in Ilford Hypam for 2 min. For quantitative autoradiography, it is important to cut down light during developing, so that this step was carried out in complete darkness. Finally, slides were washed in a running water bath for approximately 30 min, dried in an oven at 50°C, and examined under oil immersion on an Olympus microscope. In all cases the tracer used was tritium.

0.7 FEULGEN-DNA DENSITOMETRY (MICROSPECTROPHOTOMETRY)

Measurements of DNA content of individual nuclei in Feulgen-stained autoradiographs were performed on a Zeiss 02 photometer system at 570 nm. Integrated absorbance of specimens was automatically calculated by running the APAMOS MODIFIED program (Automatic Photometric Analysis of Microscopic Objects by Scanning) on a Digital PDP12 computer interfaced to the photometer. The scanning motion of the motor-driven stage sets up the measurement raster in contrast to the flying spot/static sample approach used by the Vickers M85 machines. The set-up is illustrated in Figure 0.6, and the Feulgen spectrum of a CAPT nucleus is shown in Figure 0.7, which gives confirmation that Feulgen absorbs optimally at between 550 nm and 570 nm.

0.8 COMBINED MICROSPECTROPHOTOMETRY AND QUANTITATIVE AUTORADIOGRAPHY

Two methods have been developed for quantitative estimation of silver

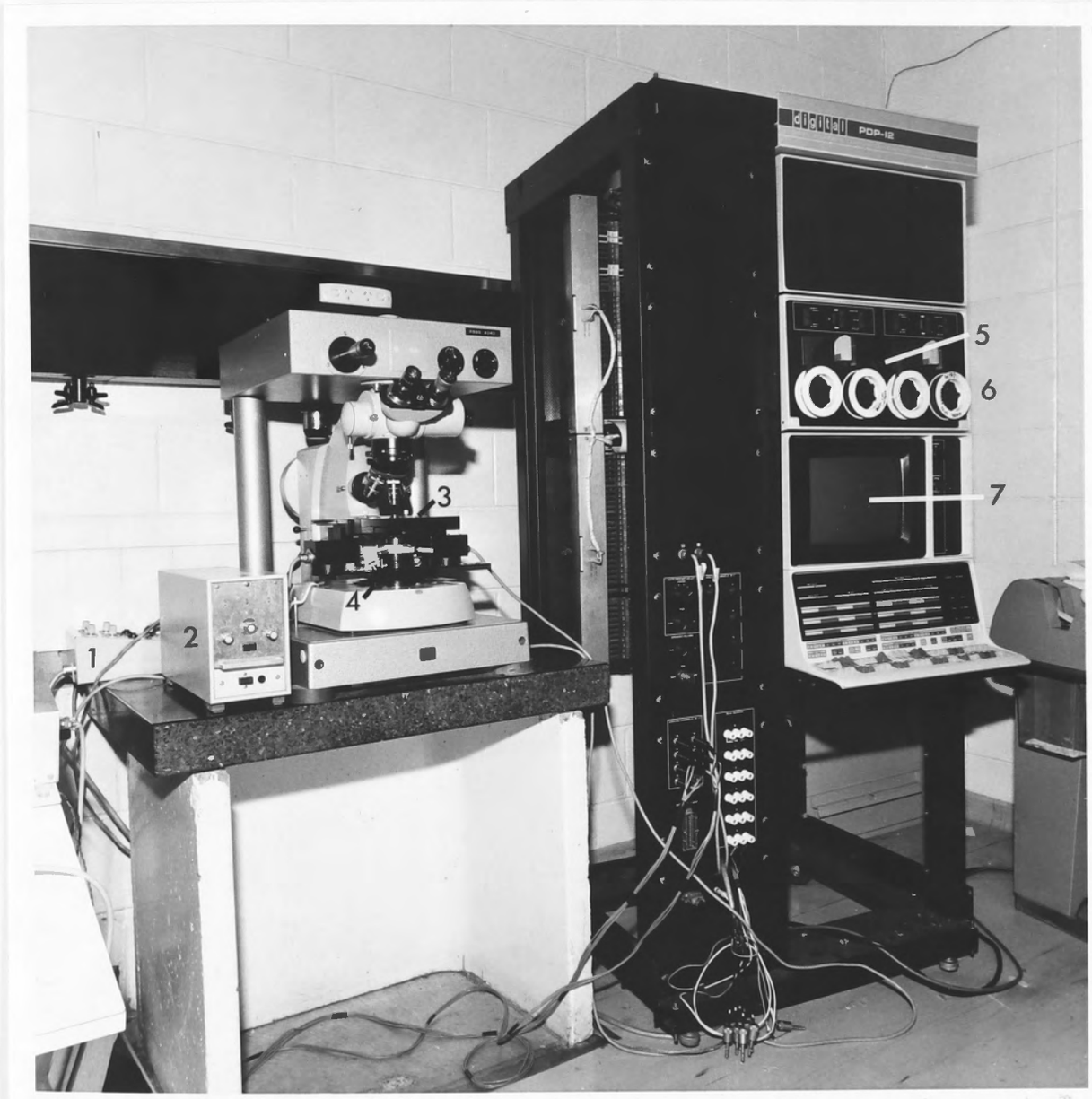


FIG. 0.6 Zeiss microspectrophotometer system interfaced to a Digital PDP computer.

(1) Stage control unit.

(2) Light intensity/sensitivity control.

(3) Motorised stage.

(4) Adjustable monochromator.

(5) Program tape (APAMOS MODIFIED).

(6) Data tape.

(7) Visual display.

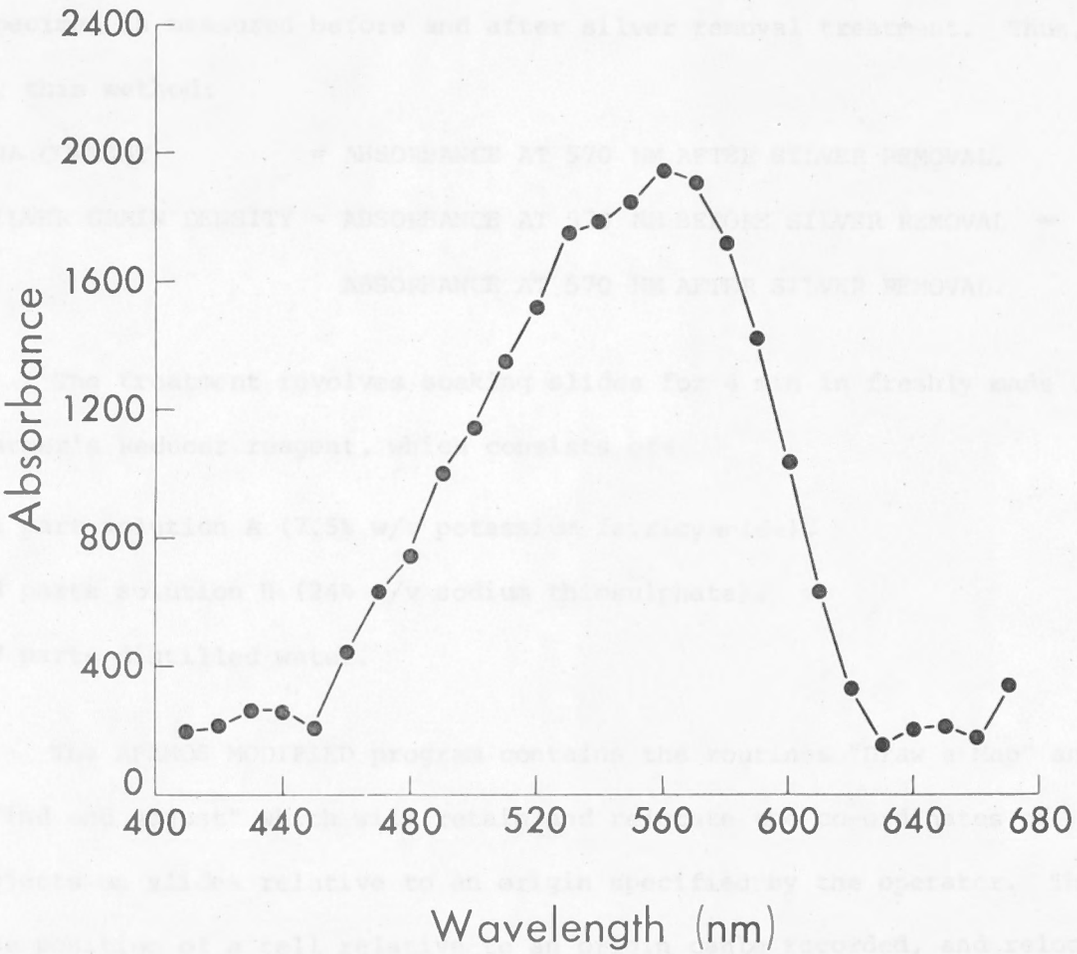


FIG. 0.7 Feulgen spectrum of a stained CAPT nucleus.

grains combined with DNA content estimation, for Feulgen stained autoradiographs of cells/protoplasts (Gould, 1979c).

(i) Silver removal method

By this method the absorbance at 570 nm of a Feulgen stained specimen is measured before and after silver removal treatment. Thus, by this method:

DNA CONTENT = ABSORBANCE AT 570 NM AFTER SILVER REMOVAL.

SILVER GRAIN DENSITY = ABSORBANCE AT 570 NM BEFORE SILVER REMOVAL − ABSORBANCE AT 570 NM AFTER SILVER REMOVAL.

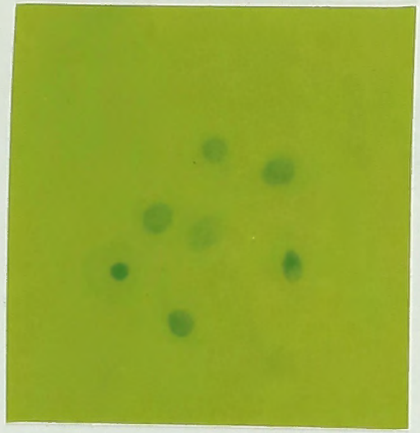
The treatment involves soaking slides for 4 min in freshly made up Farmer's Reducer reagent, which consists of:

1 part solution A (7.5% w/v potassium ferricyanide).
4 parts solution B (24% w/v sodium thiosulphate).
27 parts distilled water.

The APAMOS MODIFIED program contains the routines "Draw a Map" and "Find and Adjust" which will retain and relocate the co-ordinates of objects on slides relative to an origin specified by the operator. Thus, the position of a cell relative to an origin canbe recorded, and relocation of previously labelled cells on Feulgen stained autoradiographs can be achieved for absorbance measurements following silver removal. Fig. 0.8 shows a cell before and after Farmer's Reducer method (Fig. 0.9) shows the strong correlation (correlation coeff. = 0.968) between visual grain counting and silver density as measured by this method.

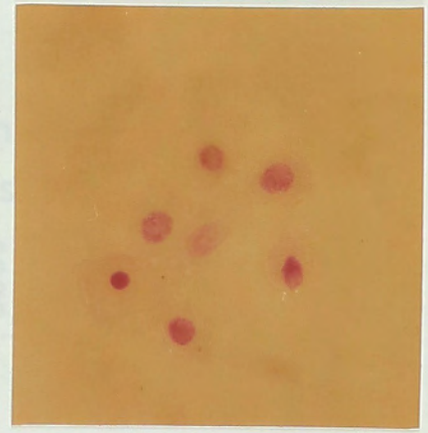
(ii) Two wave-length method

This method relies on absorption spectra differences between silver grains and Feulgen dye. It can be seen in Fig. 0.10 (after Gould, 1979c)



After silver removal.

FIG. 0.8 Feulgen stained autoradiographs of NS-1 protoplasts before and after silver removal.



After silver removal (without coloured filter).

Before silver removal.

FIG. 0.11 Feulgen stained autoradiographs of NS-1 protoplasts at various wavelengths.

670 nm 620 nm 570 nm 520 nm 470 nm

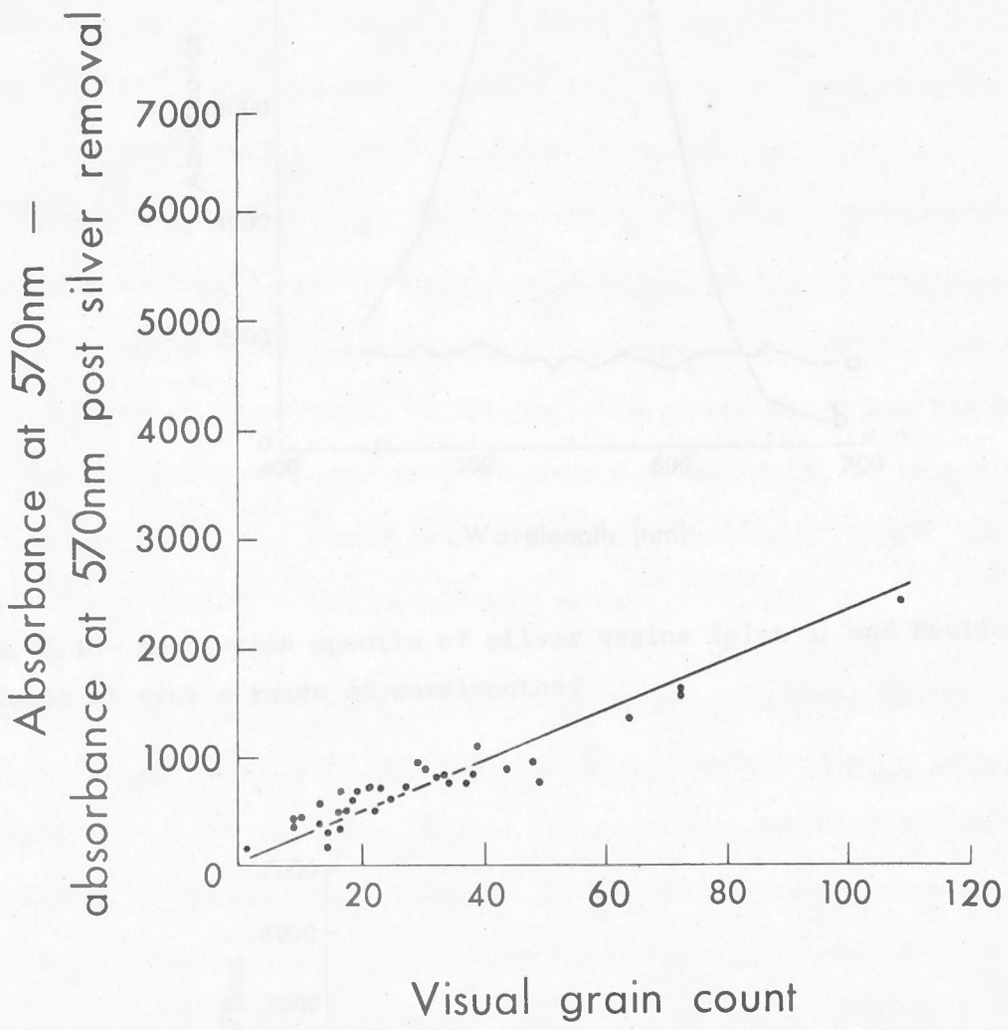


FIG. 0.9 Correlation between visual grain counts, and silver density as measured by the silver removal method of quantitative autoradiography.

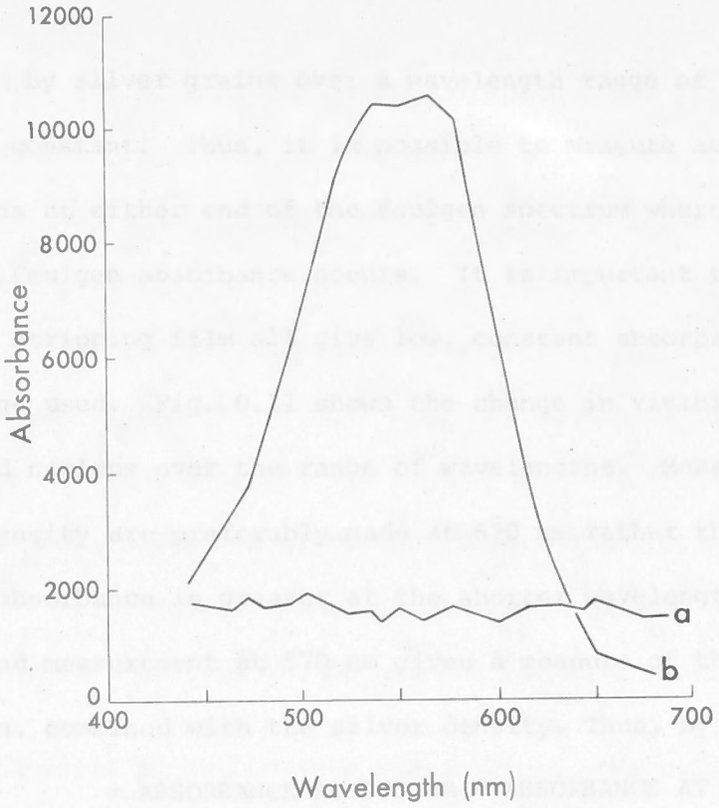


FIG. 0.10 Absorption spectra of silver grains (plot a) and Feulgen dye (plot b) over a range of wavelengths.

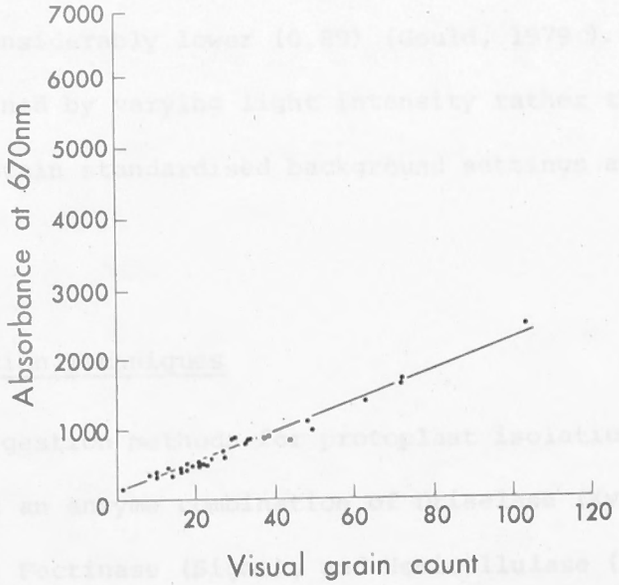


FIG. 0.12 Correlation between visual grain counts, and silver density as measured by the 2-wavelength method of quantitative autoradiography.

that absorption by silver grains over a wavelength range of 430 nm to 700 nm remains fairly constant. Thus, it is possible to measure absorbance due to silver grains at either end of the Feulgen spectrum where little interferance due to Feulgen absorbance occurs. It is important to note that DEPEX, XAM and stripping film all give low, constant absorbance over the wavelength range used. Fig. 0.11 shows the change in visibility of a Feulgen stained nucleus over the range of wavelengths. Measurements of silver grain density are preferably made at 670 nm rather than at 470 nm since Feulgen absorbance is greater at the shorter wavelength (see Fig. 0.10). A second measurement at 570 nm gives a measure of the DNA content of the specimen, combined with the silver density. Thus, by this method:

DNA CONTENT = ABSORBANCE AT 570 NM - ABSORBANCE AT 670 NM.

SILVER GRAIN DENSITY = ABSORBANCE AT 670 NM.

The correlation between visual grain counting and the quantification of silver grain density by the two wave-length method is high (correlation coefficient = 0.97) (see Fig. 0.12). Using measurements at 470 mm this coefficient is considerably lower (0.89) (Gould, 1979c). More consistent results are obtained by varying light intensity rather than detector sensitivity to obtain standardised background settings at the different wavelengths.

Protoplast Isolation Techniques

Enzymatic digestion methods for protoplast isolation were developed. It was found that an enzyme combination of Driselase (Kyowa, Hakko Kogyo Co. Ltd., Tokyo), Pectinase (Sigma), and Hemicellulase (Sigma) in a buffer of 2 mM NaH_2PO_4 plus 3 mM $CaCl_2.2H_2O$ at pH 5.5, was effective for the release of protoplasts from cell suspensions of all 3 composites, and for the NS-1 suspension cell culture. Sorbitol, at 11% or 13% was added to

osmotically buffer the protoplasts on their release, and prevent bursting. Cell suspensions were initially washed, twice, in a Protoplast Washing Buffer (P.W.B.) which consisted of 2 mM NaH_2PO_4, 3 mM $CaCl_2.2H_2O$, and either 11% of 13% sorbitol, pH adjusted to 5.5, which caused initial plasmolysis of the cells. 10 ml enzyme was added to cells from each 10 ml cell suspension, and the mixture was incubated in a sealed 9 cm petri dish, on a rotating platform (50 r.p.m.) at 27°C. The release of protoplasts was monitored under an Olympus inverted microscope. Variations in enzyme concentrations, sorbitol concentrations, and periods of incubation for each culture are documented in Table 0.5.

Once a good yield of protoplasts was obtained, the cell/enzyme mixture was filtered first through a 100 μ nylon filter, followed by a filter of smaller pore diameter (see Table 0.5), washed twice in P.W.B. and used for experimentation. Counts of the numbers of protoplasts per ml were made using a haemocytometer. The process was all carried out under sterile conditions, the enzyme mixture being pre-sterilized using a 0.45μ millipore filter.

Leaf protoplasts of both *Crepis capillaris*, and *Brachycome dichomosomatica* were obtained using the enzyme compositions and times of incubation detailed in Table 0.5. Leaf tissue was initially sterilised in 10% hypochlorite solution, for 10 min, finally sliced, and then treated as above for the cell suspensions.

Photographs of released protoplasts are shown in Figure 0.13.

TABLE 0.5 - PROTOPLAST ISOLATION METHODS

Cell Source	Enzyme Composition	Sorbitol Concentration	Incubation time (h)	Protoplast diameters (μ) (mean ± S.E.M.)	Filter size (μ)	Average % yield
CAPT	1% driselase 1% pectinase 0.5% hemicellulase	13%	12	21.2μ ± 0.157	37	30 → 50%
HA-1	4% driselase 4% pectinase 2% hemicellulase	11%	6	35 → 55	63	20 → 30%
NS-1	2% driselase 2% pectinase 1% hemicellulase	11%	3	-	63	-
LIN-31	2% driselase 2% pectinase 1% hemicellulase	11%	3 → 6	-	63	-
C. capillaris leaf	0.5% driselase 0.5% pectinase 0.025% hemicellulase	13%	12	-	63	-
B. dichromosomatica leaf	2% driselase 2% pectinase 1% hemicellulase	11%	3 → 6	-	63	-

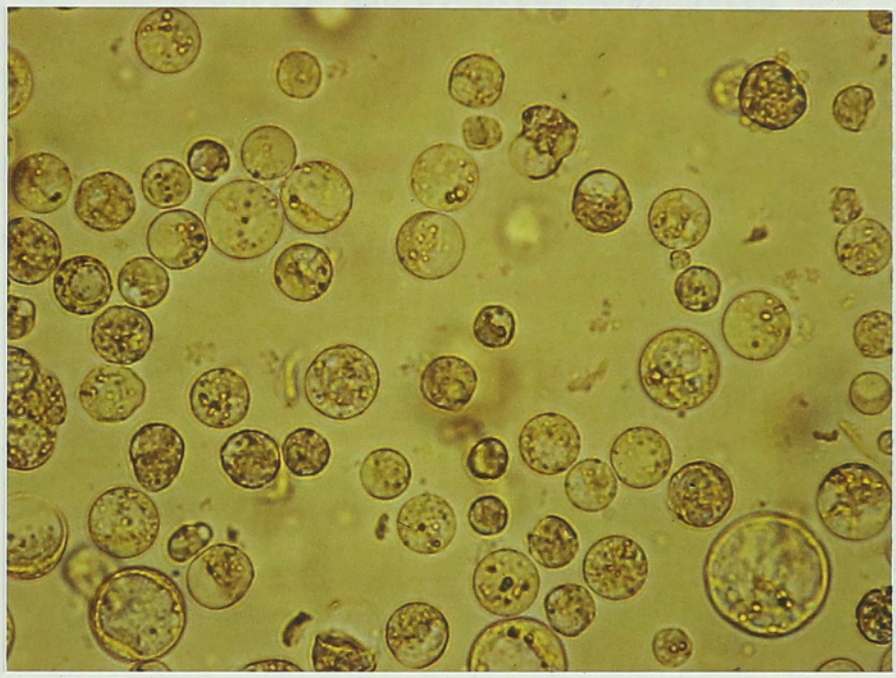


FIG. 0.13a. Protoplasts from CAPT suspension cell culture.

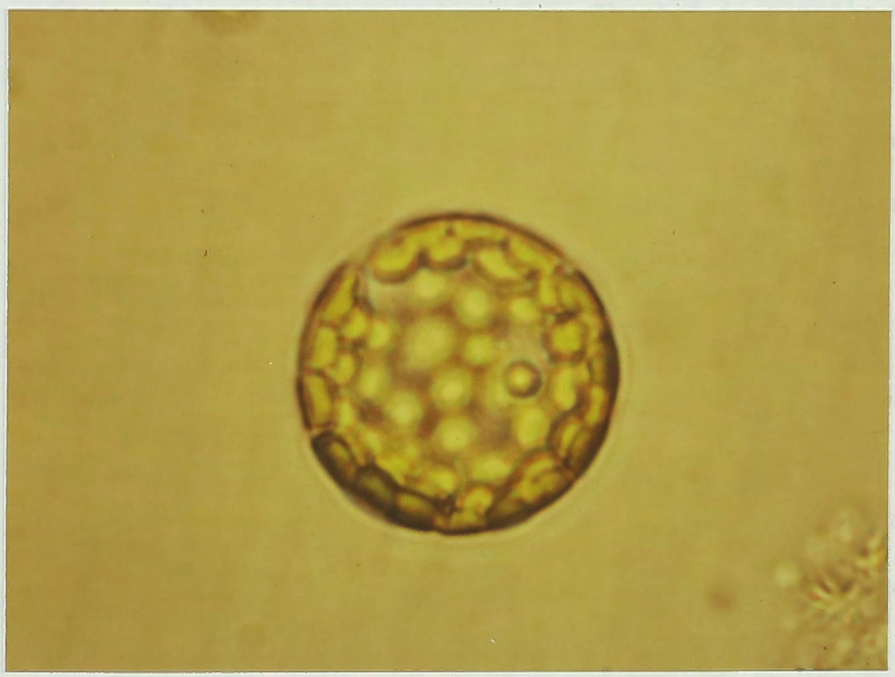


FIG. 0.13b. A leaf mesophyll protoplast of *Crepis capillaris*. Chloroplasts are clearly visible within the cytoplasm.

CHAPTER 1

CHROMOSOMAL ANALYSIS OF CELL CULTURES OF *C. capillaris*, *H. gracilis* AND *B. dichromosomatica*

1.1 INTRODUCTION

This chapter describes the chromosomal characteristics of the three species *Crepis capillaris* (2n = 6), *Haplopappus gracilis* (2n = 4), and *Brachycome dichromosomatica* (2n = 4), both in whole plant tissue and in tissue culture lines derived from these plants. Careful investigation of the organisation of the genomes of these systems is of primary importance when the proposed studies on these plants include plans to introduce exogenous genetic material into the cells. It is known that karyotypic changes, including both numerical and structural alterations, commonly occur in cells when they are removed from the whole plant and grown in a tissue culture environment (Sunderland, 1973; Bayliss, 1980). Such deviations from the normal chromosome complement must, therefore, be assessed before cells in culture are used as "hosts" in genetic modification experiments. It is difficult to analyse the consequences of such manipulations if the cells show variability in their chromosome complements or exhibit progressive karyotype evolutions. Further alterations in chromosomal make-up may also occur following the introduction of foreign genetic material. In heterokaryons (formed by fusion of two different cell types), extensive loss of chromosomes has been observed both in plant and animal systems (Gleba and Hoffmann, 1979; Weiss and Green, 1967). Again, knowledge of the numerical and structural achitecture of the original parental genomes is essential. The particular chromosomes lost can then be identified, and correlation of specific chromosome loss with any phenotypic alterations which occur at the same time, might be a starting point for genetic mapping. Such techniques are routinely employed in the

mapping of the human genome (Boone *et al.*, 1972).

Many of the plant species which have been used in fusion experiments and in studies of the uptake of foreign genetic material, have numerous, small, morphologically similar chromosomes. For example, the recent protoplast fusion experiments of Gleba and Hoffmann (1979), in which octaploid *Arabidopsis thaliana* callus protoplasts (with 40 tiny chromosomes) were fused with *Brassica campestris* protoplasts (which have 20 chromosomes), took months of hard and tedious work to analyse, and, by the authors' published admission, caused nightmares, and a determination to avoid such species in the future. Similarly, other authors have used *Nicotiana tabacum* (2n = 48), *Petunia hybrida* (2n = 14), *Glycine max* (soybean; 2n = 40), and *Arabidopsis thaliana* (2n = 10) for both fusion studies (Melchers *et al.*, 1975; Kartha *et al.*, 1974) and uptake studies (Hess, 1972; Ledoux *et al.*, 1974). Such species have been used because of the ease with which they may be manipulated in a tissue culture environment, and little emphasis has been placed on their suitability for cytogenetic work. It should be noted that, although *Arabidopsis thaliana* has a relatively low chromosome number, these chromosomes are very small and difficult to distinguish from one another morphologically.

The use of three composite species, which have only a few morphologically distinct chromosomes makes cytogenetic investigation at the single chromosome level possible in the present study.

In addition to the analysis of the gross karyotypes in these species, and their derived tissue cultures, chromosome banding techniques have been investigated. The information gained from the gross karyotype is

restricted to the description of chromosome lengths, positions of centromeres (hence the arm ratio), and the presence or absence of secondary constrictions. However, in recent years, the more sophisticated cytogenetic techniques of chromosome banding have allowed detailed description of individual chromosomes. Following treatment of dividing cells with the various banding procedures, differential staining occurs along the length of the metaphase chromosomes. Such longitudinal patterns of darkly and lightly stained regions on the chromosomes are characteristic and reproducible, thus allowing easy and more accurate identification of particular chromosomes, or parts of chromosomes, in a metaphase spread (Hsu, 1973). These banding techniques all rely on the use of Giemsa stain, which is composed of a mixture of methylene azure (which stains chromatin), eosin (which binds to azure incorporated chromatin), glycerine, and methanol, and was originally devised by Gustav Giemsa in the early 1900's to detect malaria in blood preparations. There are five major banding procedures, Q-banding (Caspersson *et al.*, 1969), C-banding (Arrighi and Hsu, 1971), G-banding (Seabright, 1971), R-banding (Dutrillaux and Lejeune, 1971), and N-banding (Matsui and Sasaki, 1973). The specific chemical reactions involved in these processes are not entirely clear, but it seems that both Q-bands and G-bands occur in A-T (adenine-thymine) rich areas of the DNA, whereas R-bands are reverse in nature to G-bands. C-banded areas correspond to sites of constitutive heterochromatin, that is repetitive DNA of any base composition, and N-bands occur at sites of nucleolus organizer regions (NOR's) (see Hsu, 1979). All these methods are routinely and successfully applied to animal cells. However, there have been far fewer reports of the use of these procedures with plants, partly because the techniques used on animal tissue often need modifications

for plant tissue. For example, it seems that G-bands have not been visualised in plants because of the high degree of contraction of plant chromatin during metaphase (Greilhuber, 1977). However, C-banding has now been reported in several plants (Schweizer, 1973; Gostev and Asker, 1979), although there have been only two studies of C-banding in plant cell cultures (Papeš *et al.*, 1978; Wochok *et al.*, 1980).

Certainly, banding techniques have proved to be invaluable in studies of somatic cell genetics in animal populations (Hashmi *et al.*, 1974), and have, in many cultures, revealed a greater degree of chromosomal heterogeneity than was previously suspected (Tiepolo and Zuffardi, 1973; Hashmi *et al.*, 1974). Similarly, the more accurate description of genome architecture provided by Giemsa banding procedures should become important in plant tissue culture. Chromosomal rearrangements, which are known to occur in cultured plant cell populations (Sacristán and Wendt-Gallitelli, 1973; Bayliss, 1980) may be readily detected, and banded chromosomes should provide cytogenetic markers, in the absence of well defined genetic loci. Although the development of well mapped genetic systems remains a prerequisite in many plant systems, a first step can be made with C-banding. It must be remembered that the regions delineated by C-banding techniques are rather crude, and may encode a few hundred genes. In this respect, polytene chromosomes can offer more refined cytogenetic markers, as has been demonstrated in the Diptera, but this naturally occurring banded effect in multistranded chromosomes has not yet been exploited in plant material, although polyteny occurs in the suspensor in some plant groups.

Regular monitoring of the chromosomal constitution of the dividing

cells in tissue culture lines of the three composite species has been carried out throughout the duration of this project. Such constant monitoring is important since it is known that not only does the karyotype often deviate from the normal diploid in cells in culture, but, also, there is commonly progressive evolution of the karyotype in the culture environment (Ford, 1964; Bayliss and Gould, 1974). Even when an apparently dominant, stable karyotype exists in a tissue culture, any slight alterations in culture regime, such as using a cell dose lower than usual for innoculation, may promote directional change in the chromosomal make-up of the population (Kaziwara, 1954). Additionally, although in many cases one characteristic karyotype may occur in the majority of cells in a culture population, in many cultures several different karyotypes may co-exist. Such chromosomal heterogeneity may be a stable situation in itself, or, alternatively, may be an intermediate state in the progressive evolution towards chromosomal homogeneity. Those populations of tissue culture cells having a single, stable, karyotype will be most useful for genetic modification experiments. For example, in populations of cells exhibiting heterogeneity of chromosome numbers per cell, it becomes difficult to analyse attempts at whole chromosome introduction into these populations. Alternatively, if a variety of different aneuploid karyotypes are represented in a population, certain of these heteroploid cells may mimic genetic changes through increased gene dosage of certain gene products, and may, then, be confused with real transformants in a gene introduction experiment.

Information gained from regular monitoring of karyotype is also of interest as an investigation of the patterns of karyotype change which occur in cultures of three related species. Such studies in plant tissue

cultures have often been restricted and unreliable, due to the use of species with large numbers of small chromosomes, so that counting may be innaccurate, and any chromosomal aberrations may go undetected. The tedious nature of cytogenetic analysis in these species may be overcome by the use of cytophotometry to look at the spread of DNA contents in either G_1 cells or G_2 cells in a population. This technique, which was used by Bayliss and Gould (1974), gives a measure of the ploidy levels in the population, but cannot yield more specific information about the numbers of chromosomes present, the type of chromosomes retained in an aneuploid situation, or the degree of morphological change in the chromosomes. Where species with low chromosome numbers have been used it has been possible to identify specific chromosomal rearrangements, and the preferential retention of certain chromosomes in an aneuploid situation (Singh *et al.*, 1972; Sacristán and Wendt-Gallitelli, 1973; Singh, 1975). In general, the information obtained about chromosome changes in plant tissue culture indicate that diploidy, polyploidy and aneuploidy all occur, as well as extensive chromosomal abnormalities in certain tissue cultures (Sacristán, 1971). In animal cell cultures a pattern of tetraploidisation, followed by loss of chromosomes to produce a stable aneuploid karyotype is commonly observed (Levan and Biesele, 1958; Hsu, 1961). The same pattern of karyotype evolution has been noted in both *Haplopappus gracilis* (Singh, 1975), and *Acer pseudoplatanus* suspension cultures (Bayliss and Gould, 1974). Certainly, there is often strong selection in plant tissue cultures for one particular karyotype to become dominant in the population (Singh *et al.*, 1972; Sacristán, 1975). Investigation of the karyotypic changes occurring in tissue cultures of three closely related species should serve to reinforce or develop previous ideas of karyotype evolution in plant tissue cultures.

Again, the use of banding techniques allows specific information about possible chromosomal rearrangements to be obtained. Comparison of banded chromosomes of tissue culture cells with the normal diploid banded karyotype of the whole plant may yield information about the possible derivation of any abnormal chromosomes.

The tumorous culture of *Crepis capillaris* (CAPT) has been included in this chromosome study, and is of particular interest because numerical and structural variations in karyotype are particularly marked in tumorous animal cell populations (Ford, 1964) and have been shown to occur more commonly in tumour derived callus cultures of *Crepis capillaris* than in the normal cultures of the same species (Sacristán and Wendt-Gallitelli, 1973). These tumorous *Crepis capillaris* cultures showed marked heterogeneity of karyotype during the first year of culture, but, after a further three years, the majority of cells in such cultured populations had a single characteristic, though abnormal, karyotpye (Sacristán, 1975), indicating analogies with the stemline concept of animal tumours (Makino, 1957). Any heterogeneity of chromosome types in an apparent stemline should be revealed by banding techniques.

1.2 MATERIALS AND METHODS

1.2.1 Orcein Staining (for gross karyotype)

Lactopropionic orcein (1% orcein in 1:1 lactic acid:propionic acid):-

Live plant material, either from cell cultures or root tips, was stained directly on a microscope slide, and gently heated by passing through a bunsen flame several times. After a few minutes the stain was

replaced with 1:1 lactopropionic acid and the cells were tapped and squashed under a cover slip ready for immediate observation under the microscope. For overnight storage coverslips were sealed with nail varnish to prevent dessication of the specimen.

Acetic orcein (1% orcein in 45% acetic acid):-

Material was treated in 0.1% colchicine for 3 h. to induce mitotic arrest and was always fixed in 3:1 ethanol:acetic acid for at least 1 h. prior to staining in acetic orcein. The fixed cells were stained in a pool of orcein on a microscope slide for 15 min. Squash preparations were then made in 45% acetic acid and observed under the microscope. Permanent preparations were made in Gurr's XAM neutral mounting medium after removal of the cover slip on dry ice (Conger and Fairchild, 1953).

1.2.2 C-banding

Slide preparation:-

After metaphase arrest in 0.1% colchicine for 3 h. and overnight fixation in 3:1 ethanol:acetic acid at 4°C, cultured cells or root tips were softened in 45% acetic acid for 1 h. before being tapped out gently onto subbed microscope slides and squashed under a coverslip. The coverslips were removed after freezing on dry ice and the slide was then washed in distilled water and left to dry for at least 24 h.

Staining procedure:-

Slides were treated with 0.2N HCl for 20 min (this step was

sometimes omitted), then washed in distilled water prior to incubation in a saturated barium hydroxide solution for 2 to 5 min. at 45°C. Immediately after removal from the barium hydroxide solution, slides were rinsed in 0.2N HCl followed by distilled water, and then left in 2 x SSC (0.3M NaCl and 0.03 M sodium citrate) for 60 min. at 65°C. After a final wash in distilled water the slides were stained in 10% Giemsa in phosphate buffer (pH 6.8) for 5 to 15 min. All slides were permanently mounted in Gurr's XAM neutral mountant.

1.2.3 Cell Cultures

The cell cultures used in this cytogenetic study are all described in the General Materials and Methods Section.

1.3 RESULTS

1.3.1 Normal Diploid Karyotypes

1.3.1.1 *Crepis capillaris*

The diploid karyotype of *C. capillaris* consists of 3 pairs of chromosomes.

(i) One pair of large (approximately 8.5 μ at metaphase) submetacentric chromosomes termed the L chromosomes.

(ii) One pair of medium sized (approximately 6 μ at metaphase) acrocentric chromosomes, satellited in the short arm, termed the SAT chromosomes.

(iii) One pair of small (approximately 4 μ at metaphase) chromosomes termed the S chromosomes.

This karyotype is represented in an idiogram in Fig. 1.1a, along with a metaphase plate of a root tip of *C. capillaris* (after Sacristán). The terminology for the 3 chromosome types is after Sacristán (1971).

1.3.1.2 *Haplopappus gracilis*

The diploid karyotype of *H. gracilis* consists of 2 pairs of chromosomes.

(i) One pair of large (approximately 8 μ at metaphase) submetacentric chromosomes, termed the A chromosomes.

(ii) One pair of smaller (approximately 6 μ at metaphase) acrocentric chromosomes, having a secondary constriction and a single satellite on the small arm, known as the B chromosomes. (Note that these are not B chromosomes in the classic sense, i.e. they are not supernumary).

When Jackson first described the karyotype of this species (1957), this was the only plant species known to have such a low chromosome number. The plant material used in this project was all derived from a variety of *H. gracilis* which shows heteromorphism in the short arm of chromosome B, one chromosome having a double satellite, whilst its' homologue retains the single satellited short arm. This variety, first described by Jackson (1963), is represented diagrammatically in Fig. 1.1b. An orcein preparation of a metaphase root tip cell, confirms the karyotypic characteristics of the idiogram (Fig. 1.1b).

1.3.1.3 *Brachycome dichromosomatica*

Plants of *Brachycome dichromosomatica* (formerly included in

B. lineariloba, Race A, see Carter, 1978) were collected, in the field, at Wild Dog Glen, South Australia, and two distinct karyotypic types were obtained. Both chromosomal types are included in *Brachycome dichromosomatica* var. *dichromosomatica* one being deme 2, and the other deme 4 (see Carter, 1978). These chromosome complements, each consist of 2 pairs of chromosomes and this low chromosome number for an Australian species was first reported, in 1968, by Smith-White. The characteristics of the chromosomes of the two demes are as follows:

Deme 2:

(i) One pair of large (approximately 8 μ at metaphase) submetacentric chromosomes, termed chromosome I.

(ii) One pair of smaller (approximately 6 μ at metaphase) submetacentric chromosomes, satellited in the short arm, termed chromosome II.

Deme 4:

(i) One pair of large (approximately 8 μ at metaphase) submetacentric chromosomes, with a secondary constriction in the longer arm, termed chromosome I.

(ii) One pair of smaller (approximately 6 μ at metaphase) submetacentric chromosomes, satellited in the short arm, termed chromosome II.

Idiograms of both karyotypes are shown in Fig. 1.1c and d, along with examples of metaphase cells from root tips of these plants.

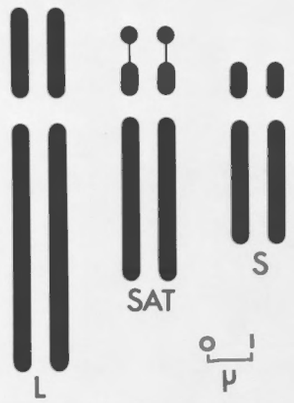
FIG. 1.1 (a) The normal diploid karyotype of *Crepis capillaris* in the form of an idiogram, and as seen in a root tip metaphase preparation (after Sacristán and Wendt-Galitelli, 1971).

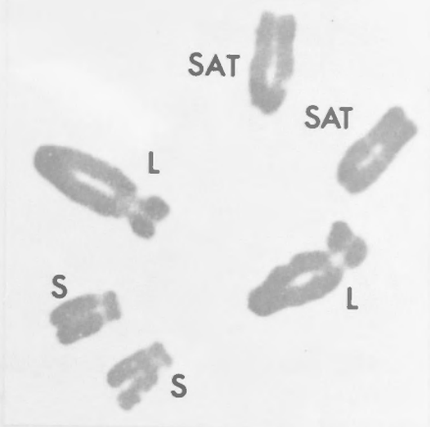
(b) The normal diploid karyotype of *Haplopappus gracilis* in the form of an idiogram, and as seen in an orcein stained metaphase cell of a root tip.

(c) The normal diploid karyotype of *Brachycome dichromosomatica* var. dichromosomatica deme 2, in the form of an idiogram and as seen in an orcein stained root tip metaphase cell.

(d) The diploid karyotype of *Brachycome dichromosomatica* var. dichromosomatica deme 4, in the form of an idiogram, and as seen in a root tip metaphase cell (after Watanabe *et al.*, 1975).

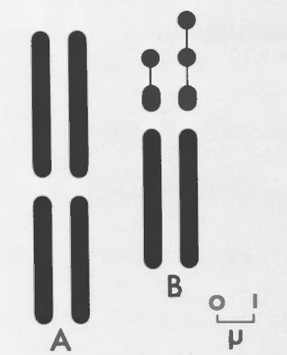
a

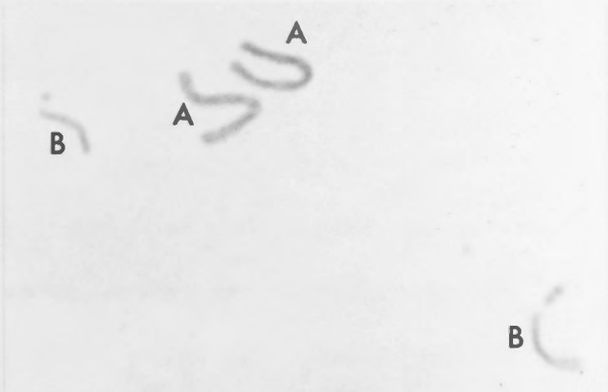




Crepis capillaris

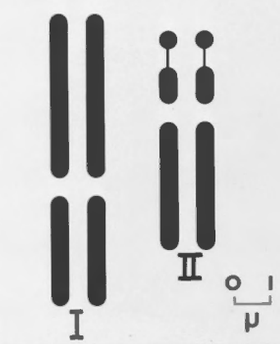
b





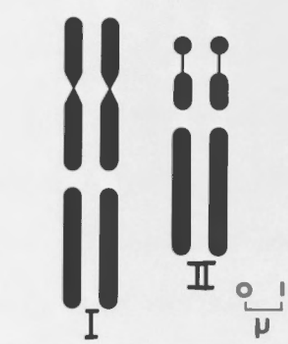
Haplopappus gracilis

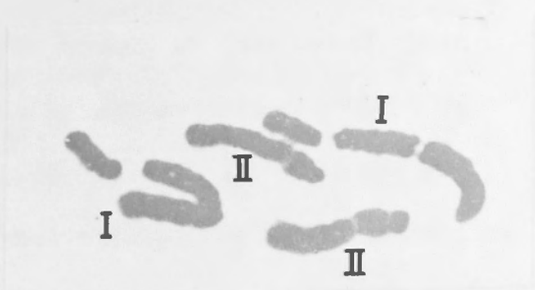
c



Brachycome dichromosomatica
var. dichromosomatica. deme 2

d





var. dichromosomatica. deme 4

1.3.2 Karyotypic Analysis of Cell Cultures

1.3.2.1 *Crepis capillaris*

CAP culture:-

The normal, diploid, 6 chromosome karyotype was seen in this non-tumorous callus culture (Fig. 1.2a).

CAPT callus and suspension cell culture:-

The CAPT culture is a tumorous cell line of *Crepis capillaris* which was originally induced with *Agrobacterium tumefaciens* (Sacristán and Melchers, 1970). The culture was received from Sacristán in the form of a diploid callus culture. After 3 years of culture 7-chromosome karyotypes had arisen in 25% of the callus population and a few tetraploid (i.e. 12 chromosome) cells were seen (Fig. 1.2b). The suspension cell culture derived from this callus had a 7-chromosome complement in the majority of dividing cells after one year of culture (Fig. 1.2c), and during a further year the 7 chromosome karyotype remained in almost 100% of the dividing population (Fig. 1.2d, e). Fig. 1.2f shows the chromosome numbers in a culture which was sub-cultured every 7 days with a 20 ml innoculum of cells rather than a 10 ml innoculum. It can be seen that this culture also has a 7 chromosome complement in the majority of the dividing population. It should be noted, at this point, that monitoring of chromosome number can only be achieved in the dividing population and this may not necessarily be representative of the whole population. It may be, for instance, that tetraploids traverse mitosis more slowly than diploids so that the fraction of tetraploids in the mitotic population is greater than in the population as a whole. However,

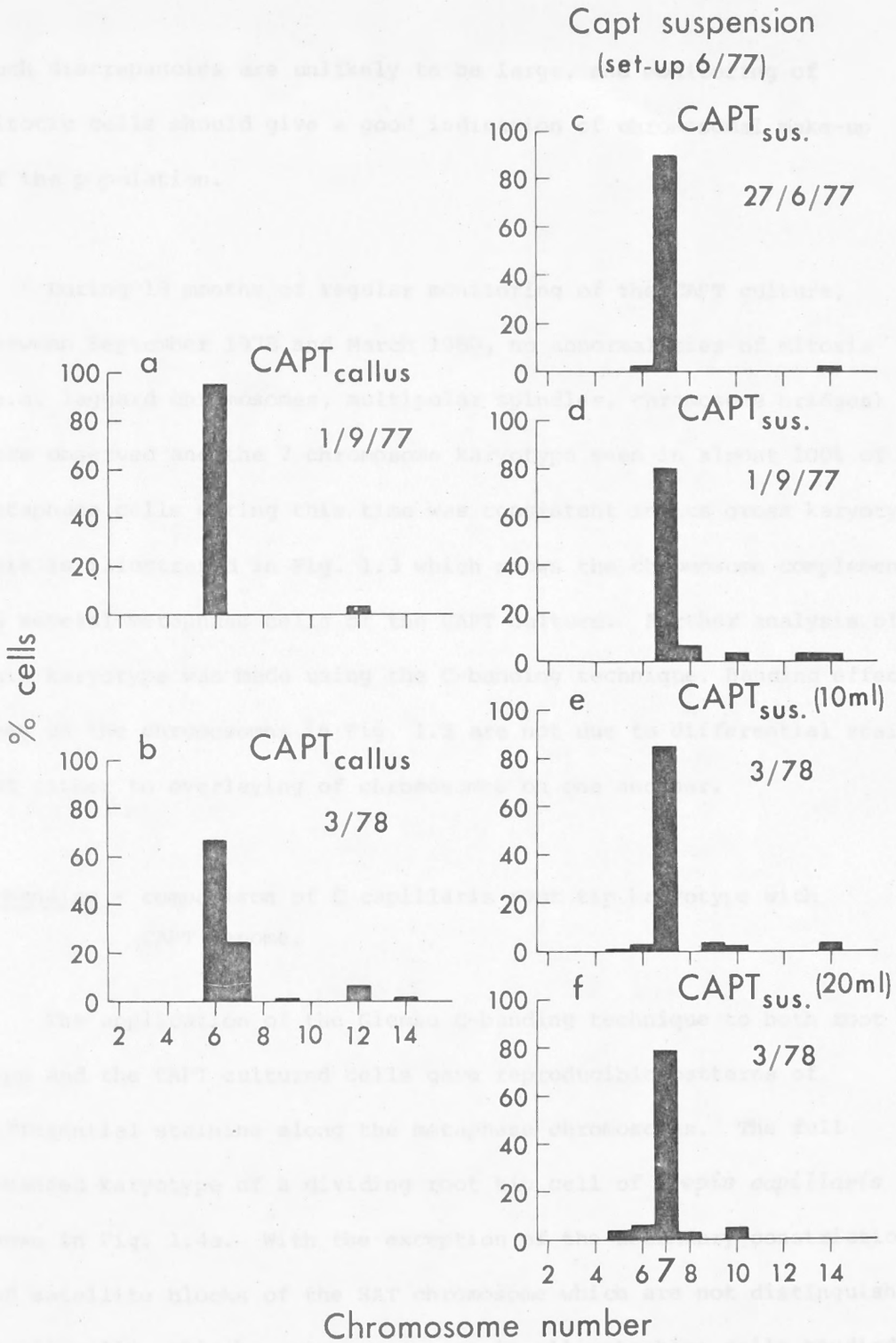


FIG. 1.2 Frequency distribution of chromosome numbers observed in dividing cells of (a) CAP callus (23 counts in Sept. 1977); (b) CAPT callus (100 counts in March 1978); (c) CAPT suspension (80 counts on 27/6/77); (d) CAPT suspension (77 counts on 1/9/1977); (e) CAPT suspension (100 counts in March 1978); (f) CAPT suspension, 20 ml innoculum (100 counts, March 1978).

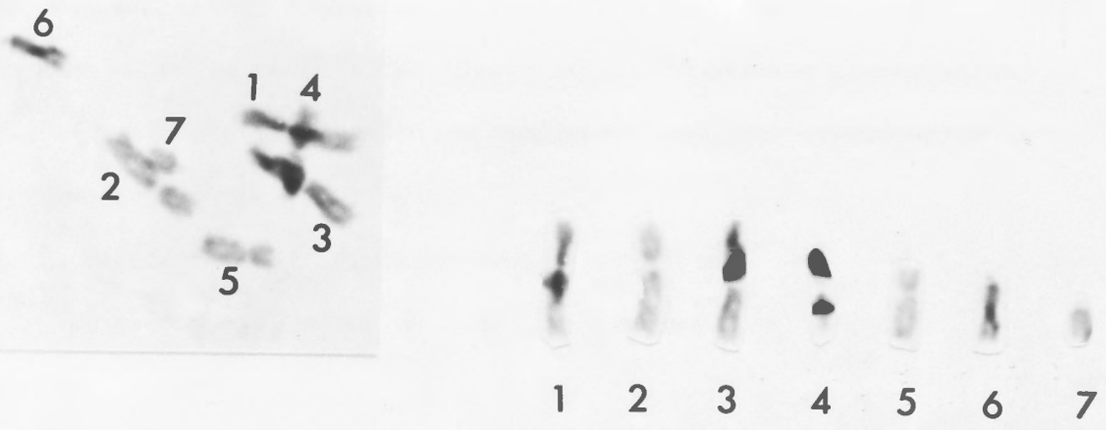
such discrepancies are unlikely to be large, and monitoring of mitotic cells should give a good indication of chromosomal make-up of the population.

During 18 months of regular monitoring of the CAPT culture, between September 1978 and March 1980, no abnormalities of mitosis (e.g. laggard chromosomes, multipolar spindles, chromosome bridges) were observed and the 7 chromosome karyotype seen in almost 100% of metaphase cells during this time was consistent in its gross karyotype. This is illustrated in Fig. 1.3 which shows the chromosome complement in several metaphase cells of the CAPT culture. Further analysis of this karyotype was made using the C-banding technique. Banding effects seen on the chromosomes in Fig. 1.3 are not due to differential staining but rather to overlaying of chromosomes on one another.

C-banding - comparison of C capillaris root tip karyotype with CAPT genome.

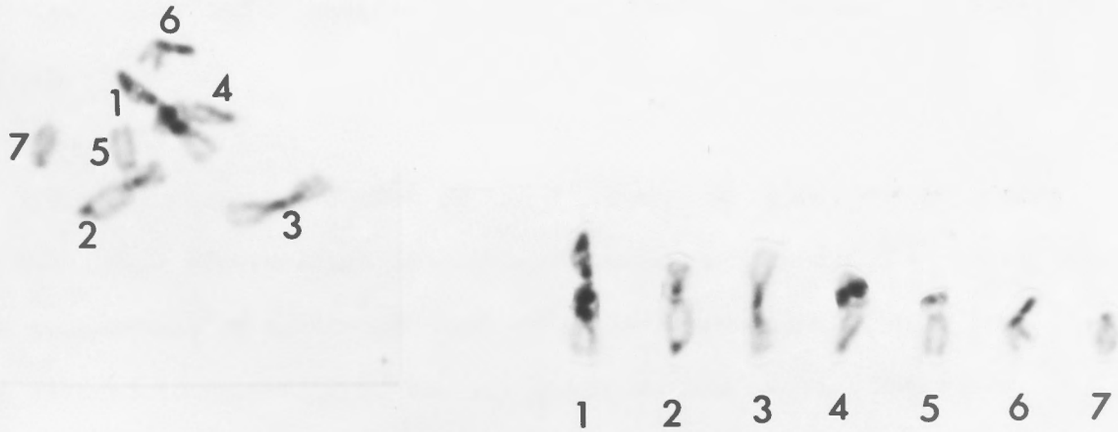
The application of the Giemsa C-banding technique to both root tips and the CAPT cultured cells gave reproducible patterns of differential staining along the metaphase chromosomes. The full C-banded karyotype of a dividing root tip cell of *Crepis capillaris* is shown in Fig. 1.4a. With the exception of the secondary constriction and satellite blocks of the SAT chromosome which are not distinguishable in all cells, this karyotype was seen in all metaphase cells studied. Fig. 1.4b presents C-banded chromosomes, seen in 6 different cells. Although all 6 chromosomes of the diploid complement are not seen in each of these cells, only 3 chromosome types (L, SAT, S) are observed, with consistent patterns of C-banding. Mean chromosome lengths (25

FIG. 1.3 Three Feulgen stained metaphase cells (a, b and c) of CAPT suspension culture, each showing the same 7 chromosome types. The darkened bands displayed on some of the "cut-out" chromosomes are the results of overlaying of the chromosomes in the metaphase preparations.



a

b



c

measurements for each chromosome type), obtained by direct measurement under the microscope (Table 1.1) have allowed construction of the idiograms shown in this table. There are 20 C-positive blocks which are always present in the diploid complement and four others which are only seen in some cells, namely:-

(i) The centromeric block on each L chromosome.

(ii) The telomeric block on each L chromosome.

The mean total length of the genome at metaphase, is 36.98 μ (by addition of the individual chromosome lengths), of which 11.02 μ is C-band positive and therefore assumed to be heterochromatic in nature. Thus, the mean fraction of heterochromatin in the genome is 0.298 (see Table 1.3)

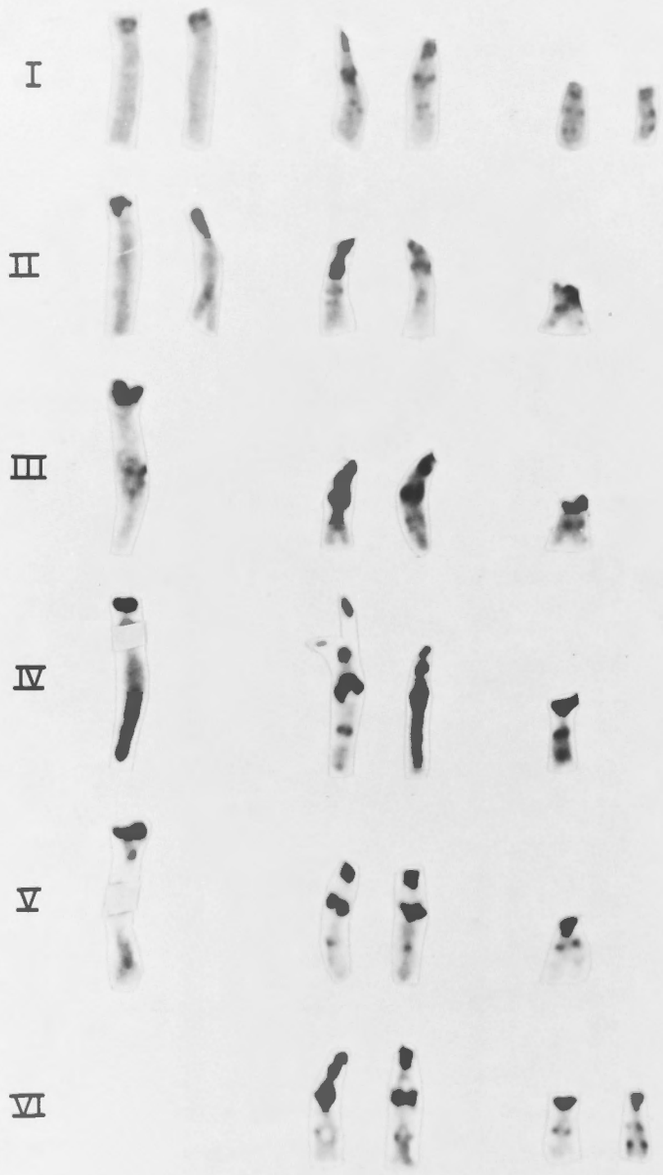
Although only a small number of complete C-banded metaphase plates of the CAPT culture were observed (due to dispersion of the chromosomes during the tapping and squashing procedure), an exhaustive search of several C-banded preparations, which were produced over a period of six months, has revealed only seven chromosome types, with consistent patterns of C-banding. Fig. 1.5b shows such banded chromosomes from several cells, and 2 examples of complete banded metaphases are displayed in Fig. 1.5a.

The mean lengths of each of the 7 chromosome types, obtained from 25 individual measurements on each chromosome, are recorded in Table 1.2, and measurement of the C-band regions of 10 chromosomes of each type has allowed construction of the idiograms in this table. There are 26 C-positive blocks consistently visible in the CAPT genome, whilst

FIG. 1.4 (a) Full C-banded karyotype of *Crepis capillaris* root tip.

(b) The chromosome sets (some incomplete metaphases), numbered I → VI are from 6 different C-banded metaphase cells of *Crepis capillaris* root tip, and can be classified into 3 different chromosomal types, L, SAT and S.

a



b

TABLE 1.1 - C-BANDED KARYOTYPE OF *C. capillaris* ROOT TIP

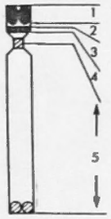
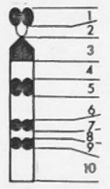
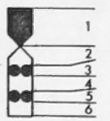
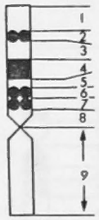
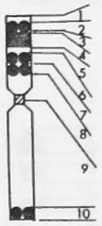
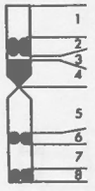
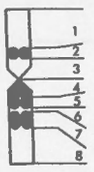
	Sections	1	2	3	4	5	6	7	8	9	10	Total C-band material
	CHROMOSOME L = 8.45μ ± 0.22 (mean of 25 measurements)											
	Relative lengths of each section (means of 9 chromosomes) ± S.E.M.	0.075 ±0.006	0.0365 ±0.0023	0.0385 ±0.0028	0.0674 ±0.0059	0.783 ±0.0077						0.113 ±0.0088
	Actual lengths (μ) (taking mean length = 8.45μ) ± S.E.M.	0.634 ±0.05	0.308 ±0.019	0.325 ±0.023	0.569 ±0.05	6.616 ±0.065						0.959 ±0.073
	CHROMOSOME SAT = 6.32μ ± 0.22 (mean of 25 measurements)											
	Relative lengths of each section (means of 10 chromosomes) ± S.E.M.	0.094 ±0.0065	- -	0.096 ±0.0094	0.12 ±0.0071	0.13 ±0.083	0.17 ±0.013	0.072 ±0.004	0.047 ±0.0055	0.061 ±0.0067	0.205 ±0.019	0.453 ±0.11
	Actual lengths (μ) (taking mean length = 6.3μ) ± S.E.M.	0.59 ±0.041		0.61 ±0.059	0.77 ±0.045	0.84 ±0.052	1.1 ±0.082	0.46 ±0.025	0.30 ±0.034	0.39 ±0.04	1.3 ±0.12	2.89 ±0.22
	CHROMOSOME S = 3.72μ ± 0.09 (mean of 25 measurements)											
	Relative lengths of each section (means of 8 chromosomes) ± S.E.M.	0.202 ±0.0066	0.145 ±0.012	0.129 ±0.0075	0.158 ±0.012	0.116 ±0.0075	0.161 ±0.019					0.447 ±0.022
	Actual lengths (μ) (taking mean length = 3.72μ) ± S.E.M.	0.75 ±0.025	0.541 ±0.044	0.478 ±0.028	0.586 ±0.045	0.433 ±0.028	0.6 ±0.069					1.66 ±0.081

FIG. 1.5 (a) Two examples of the full C-banded karyotype in the CAPT culture.

(b) The chromosome sets (some incomplete metaphases) numbered I → IX are from 9 different C-banded metaphase cells of the CAPT culture, and can be classified into 7 different chromosomal types.

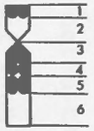
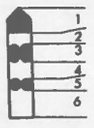

4
3
1
6
7
5
2
5
6
7
1
3
2
4
a
I
II
III
IV
V
VI
VII
VIII
IX
b
1
2
3
4
5
6
7

TABLE 1.2 - C-BANDED KARYOTYPE OF CAPT

Sections	1	2	3	4	5	6	7	8	9	10	Total C-band material
 CHROMOSOME 1 = 8.01μ ± 0.2 (mean of 25 measurements)											
Relative lengths of each section (means of 10 chromosomes) ± S.E.M.	0.123 ±0.01	0.055 ±0.0034	0.09 ±0.0077	0.11 ±0.005	0.033 ±0.008	0.054 ±0.0035	0.062 ±0.0035	0.045 ±0.0091	0.429 ±0.0084		0.281 ±0.015
Actual lengths (μ) (taking mean length = 8.01μ) ± S.E.M.	0.99 ±0.08	0.44 ±0.027	0.72 ±0.062	0.88 ±0.04	0.26 ±0.064	0.43 ±0.028	0.5 ±0.028	0.36 ±0.073	3.44 ±0.067		2.25 ±0.12
 CHROMOSOME 2 = 7.74μ ± 0.19 (mean of 25 measurements)											
Relative lengths of each section (means of 10 chromosomes) ± S.E.M.	0.037 ±0.0089	0.037 ±0.0066	0.011 ±0.0011	0.072 ±0.0067	0.038 ±0.0027	0.053 ±0.002	0.069 ±0.0035	0.085 ±0.005	0.546 ±0.013	0.057 ±0.0038	0.288 ±0.023
Actual lengths (μ) (taking mean length = 7.74μ) ± S.E.M.	0.29 ±0.069	0.29 ±0.051	0.09 ±0.087	0.54 ±0.052	0.29 ±0.021	0.41 ±0.015	0.53 ±0.027	0.66 ±0.039	4.23 ±0.1	0.44 ±0.029	2.21 ±0.17
 CHROMOSOME 3 = 6.53μ ± 0.17 (mean of 25 measurements)											
Relative lengths of each section (means of 10 chromosomes) ± S.E.M.	0.11 ±0.0065	0.129 ±0.0063	0.114 ±0.008	0.12 ±0.006	0.238 ±0.01	0.079 ±0.0032	0.148 ±0.0077	0.068 ±0.0034			0.396 ±0.019
Actual lengths (μ) (taking mean length = 6.56μ) ± S.E.M.	0.72 ±0.043	0.85 ±0.041	0.75 ±0.052	0.79 ±0.039	1.56 ±0.066	0.52 ±0.021	0.97 ±0.051	0.45 ±0.022			2.61 ±0.12
 CHROMOSOME 4 = 5.73μ ± 0.15 (mean of 25 measurements)											
Relative lengths of each section (means of 10 chromosomes) ± S.E.M.	0.26 ±0.0094	0.11 ±0.0046	0.089 ±0.0033	0.067 ±0.0035	0.08 ±0.0041	0.012 ±0.0063	0.14 ±0.025	0.25 ±0.074			0.397
Actual lengths (μ) (taking mean length = 5.73μ) ± S.E.M.	1.45 ±0.054	0.61 ±0.026	0.51 ±0.019	0.38 ±0.02	0.46 ±0.023	0.071 ±0.036	0.83 ±0.14	1.43 ±0.042			2.28

continued

TABLE 1.2 - continued

	Sections	1	2	3	4	5	6	7	8	9	10	Total C-band material
	CHROMOSOME 5 = 4.23μ ± 0.08 (mean of 25 measurements)											
	Relative legnths of each section (means of 10 chromosomes) ± S.E.M.	0.16 ±0.0092	0.12 ±0.0057	0.10 ±0.0098	0.12 ±0.0065	0.20 ±0.011	0.29 ±0.015					0.58 ±0.037
	Actual lengths (μ) (taking mean length = 4.23μ) ± S.E.M.	0.67 ±0.04	0.52 ±0.024	0.44 ±0.041	0.51 ±0.028	0.85 ±0.048	1.24 ±0.062					2.47 ±0.16
	CHROMOSOME 6 = 4.28μ ± 0.11 (mean of 25 measurements)											
	Relative lengths of each section (means of 10 chromosomes) ± S.E.M.	0.16 ±0.009	0.14 ±0.013	0.16 ±0.012	0.18 ±0.0067	0.098 ±0.0045	0.27 ±0.012					0.418 ±0.026
	Actual lengths (μ) (taking mean length = 4.28μ) ± S.E.M.	0.68 ±0.038	0.6 ±0.055	0.67 ±0.051	0.78 ±0.029	0.42 ±0.019	1.14 ±0.057					1.77 ±0.11
	CHROMOSOME 7 = 2.72μ ± 0.08 (mean of 25 measurements)											
	Relative lenghts of each section (means of 10 chromosomes) ± S.E.M.	0.27 ±0.0092	0.20 ±0.011	0.30 ±0.015	0.23 ±0.013							0.57 ±0.024
	Actual lengths (μ) (taking mean length = 2.72μ) ± S.E.M.	0.73 ±0.025	0.55 ±0.03	0.81 ±0.41	0.63 ±0.035							1.54 ±0.066

the centromeric block on chromosome 2 is not always seen.

By addition of the individual chromosome lengths, the mean total length of the CAPT genome has been calculated to be 39.24 μ which is slightly greater than that of the normal root tip karyotype (36.98 μ). Additionally, the mean DNA content of the 100 G_1 cells of the CAPT culture (1827 ± 28.5 arbitrary units), as measured by Feulgen microspectrophotometry, is considerably greater (1.3 times) than the mean DNA content of 100 G_1 cells of the root tip (1416 ± 19.2 arbitrary units), (see Table 1.3). Thus, the cultured aneuploid cells not only have a hyperdiploid chromosome number, but also have much more DNA than the normal 6-chromosome diploid karyotype. Since the total DNA content of the CAPT cells is considerably increased, whereas the total chromatin length is very close to the root tip cells, it follows that in the cultured cells there must be significantly more DNA packed into each unit length of chromatin. From Table 1.3 it can be seen that the mean DNA content per micron of the CAPT genome is 46.6 arbitrary units, as compared with 38.3 arbitrary units for the root tip. Whether the DNA is in general more tightly packed in the CAPT genome, or whether there is simply a greater proportion of heterochromatin (which contains DNA in a tightly packed form) than in the root tip cells is not clear from this data alone.

The mean fraction of C-band material (heterochromatin) per CAPT genome is 0.386, which is 1.3 times greater than in the root tip. It was previously noted that the DNA content of the CAPT genome is also 1.3 times greater than in the root tip. Thus, it seems that this "extra" DNA in CAPT cells is entirely heterochromatic, and is concentrated into the C-band regions of the genome.

TABLE 1.3 - SUMMARY OF C-BANDING ON *C. capillaris* ROOT TIP AND CAPT CULTURE.

CHROMOSOME TYPE	Root Tip			Culture						
	L	SAT	S	1	2	3	4	5	6	7
Mean chromosome length (μ) (25 measurements of each) ± S.E.M.	8.45±0.22	6.32±0.16	3.72±0.09	8.01±0.2	7.74±0.19	6.53±0.17	5.73±0.15	4.23±0.08	4.28±0.11	2.72±0.08
Mean total genome length		36.98±0.47					39.24±0.98			
Mean C-band length (μ) per chromosome (8 to 10 chromosomes of each ± S.E.M.	0.96±0.073	2.89±0.22	1.66±0.11	2.25±0.12	2.21±0.17	2.61±0.14	2.28±0.21	2.47±0.16	1.77±0.11	1.54±0.07
Mean C-band length (μ) per genome ± S.E.M.		11.02±0.40					15.13±0.98			
Mean fraction C-band material per genome		0.298					0.386			
Mean G1 DNA content (100 measurements) ± S.E.M.		1416±19.2					1827±28.5			
Number of C-bands per genome		20 + 4 variable					26 + 1 variable			
Mean DNA content per micron of chromatin		38.3					46.6			

FIG. 1.6 (a) C-banded metaphase of a *Crepis capillaris* root tip cell.

(b) Idiogram of the C-banded karyotype of *Crepis capillaris* root tip. Below the idiogram representative examples of each chromosome type are shown, along with mean lengths, in microns, of 8 to 10 chromosomes of each type.

(c) C-banded metaphase of a CAPT cell.

(d) Idiogram of the C-banded karyotype of the CAPT cell line. Below the idiogram, representative examples of each chromosome type are shown along with mean lengths, in microns, of 10 chromosomes of each type.

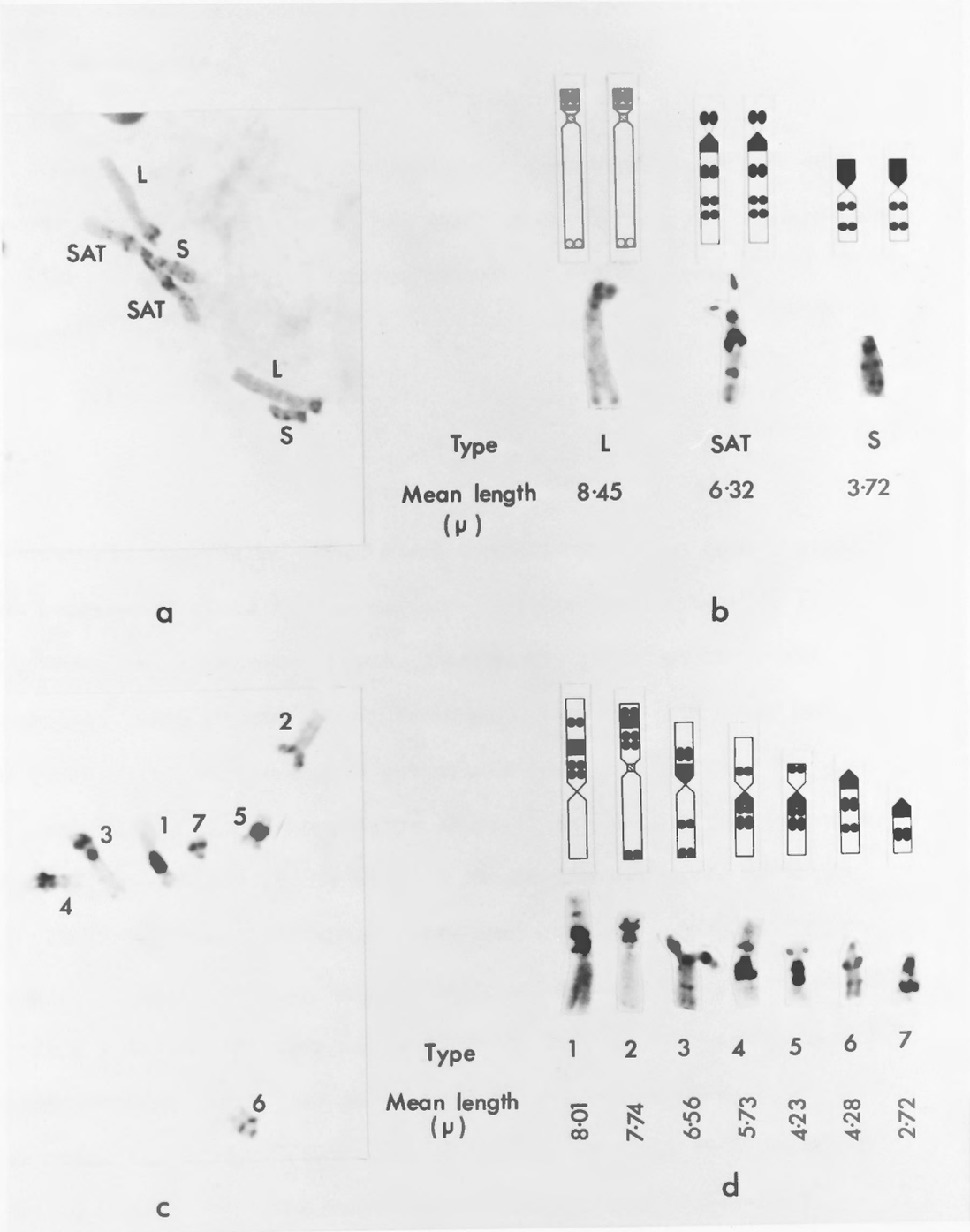

L
SAT
S
SAT
L
S
a
Type
L
SAT
S
Mean length (μ)
8·45
6·32
3·72
b
2
3
1
7
5
4
6
c
Type
1
2
3
4
5
6
7
Mean length (μ)
8·01
7·74
6·56
5·73
4·23
4·28
2·72
d

Fig. 1.6 is a composite diagram, summarising the characteristics of the C-banded root tip karyotype as compared with the CAPT chromosome complement. Table 1.3 contains the quantitative data obtained from this comparative analysis.

It should also be noted that there is a greater proportion of metacentric type chromosomes in the CAPT genome (5 out of 7 chromosomes) as compared with the normal diploid (4 out of 6 chromosomes).

1.3.2.2 *Haplopappus gracilis*

HA-1:-

This cell suspension culture was originally set up from a diploid callus culture (derived from a culture initiated by Erikson in 1967). The culture was maintained in this laboratory, prior to the start of this project, with regular 4-day transfers, for a period of 14 months. After 2 months of culture, some tetraploid cells had arisen, representing approximately 30% of the population (Fig. 1.7a), and, after a further 3 months, 85% of the population had a 6 chromosome aneuploid karyotype (Fig. 1.7b). In June 1977 the present author began transferring this culture at 7 day intervals, and 3 months later the cells predominantly retained a 6 chromosome karyotype, although 10% of the population had 12 chromosomes per cell, and there were a few cells with 8 or 11 chromosomes (Fig. 1.7c). Seven months later, in April 1978, although 57% of the population had 6 chromosomes per cell, a considerable proportion (12%) now had a 7 chromosome karyotype and 12% of the cell population had 12 chromosomes per cell (Fig. 1.7d). By April 1980, 2 years later, the 6 chromosome karyotype had been lost from the culture,

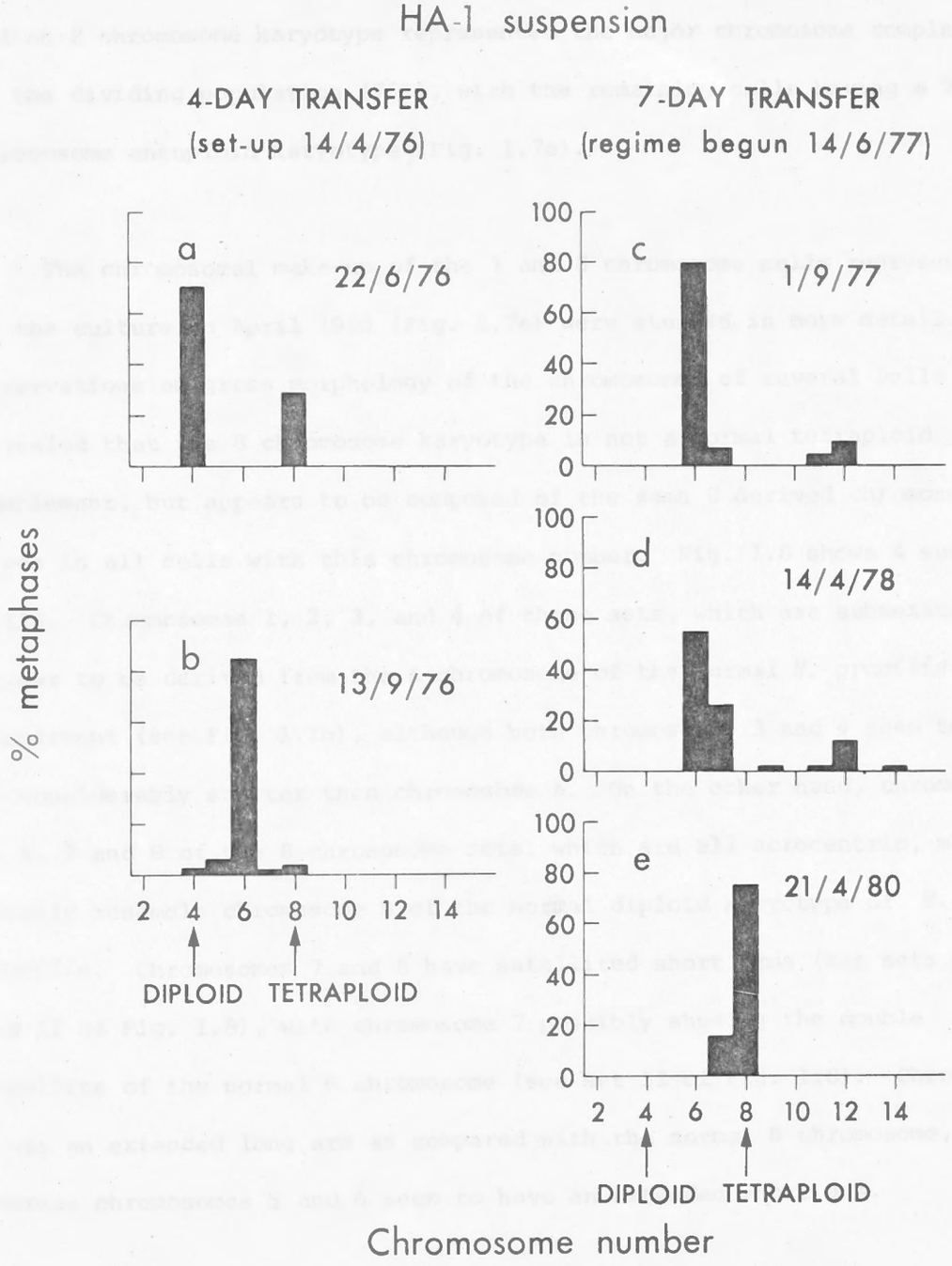


FIG. 1.7 Frequency distribution of chromosome numbers observed in dividing cells of the HA-1 suspension cell culture (a) 4-day transfer regime, 38 counts on 22/6/76; (b) 4-day transfer regime, 117 counts on 13/9/76; (c) 7-day transfer regime, 29 counts on 1/9/1977; (d) 7-day transfer regime, 49 counts on 14/4/78; (e) 7-day transfer regime, 89 counts on 21/4/80.

and an 8 chromosome karyotype represented the major chromosome complement in the dividing population (75%), with the remaining cells having a 7 chromosome aneuploid karyotype (Fig. 1.7e).

The chromosomal make-up of the 7 and 8 chromosome cells represented in the culture in April 1980 (Fig. 1.7e) were studied in more detail. Observations on gross morphology of the chromosomes of several cells revealed that the 8 chromosome karyotype is not a normal tetraploid complement, but appears to be composed of the same 8 derived chromosome types in all cells with this chromosome number. Fig. 1.8 shows 4 such cells. Chromosomes 1, 2, 3, and 4 of these sets, which are submetacentric, appear to be derived from the A chromosome of the normal *H. gracilis* complement (see Fig. 1.1b), although both chromosomes 3 and 4 seem to be considerably shorter than chromosome A. On the other hand, chromosomes 5, 6, 7 and 8 of the 8 chromosome sets, which are all acrocentric, more closely resemble chromosome B of the normal diploid karyotype of *H. gracilis*. Chromosomes 7 and 8 have satellited short arms (see sets I and II of Fig. 1.8), with chromosome 7 possibly showing the double satellite of the normal B chromosome (see set II of Fig. 1.8). Chromosome 7 has an extended long arm as compared with the normal B chromosome, whereas chromosomes 5 and 6 seem to have an extended short arm.

Three examples of 7 chromosome complements are shown in Fig. 1.9. From these examples alone the consistency of the karyotype in all cells having 7 chromosomes is uncertain. In fact there seem to be some discrepancies of gross morphology in the chromosomes of the 3 illustrated examples *e.g.* cell I has 3 large chromosomes and 4 small chromosomes, whereas cell III has 4 (maybe 5) large chromosomes and 3 (or 2) small

FIG. 1.8 I → IV show metaphase plates for 4 different cells of the HA-1 culture, all having the same 8 chromosome types, as indicated on the right-hand side of the figure.

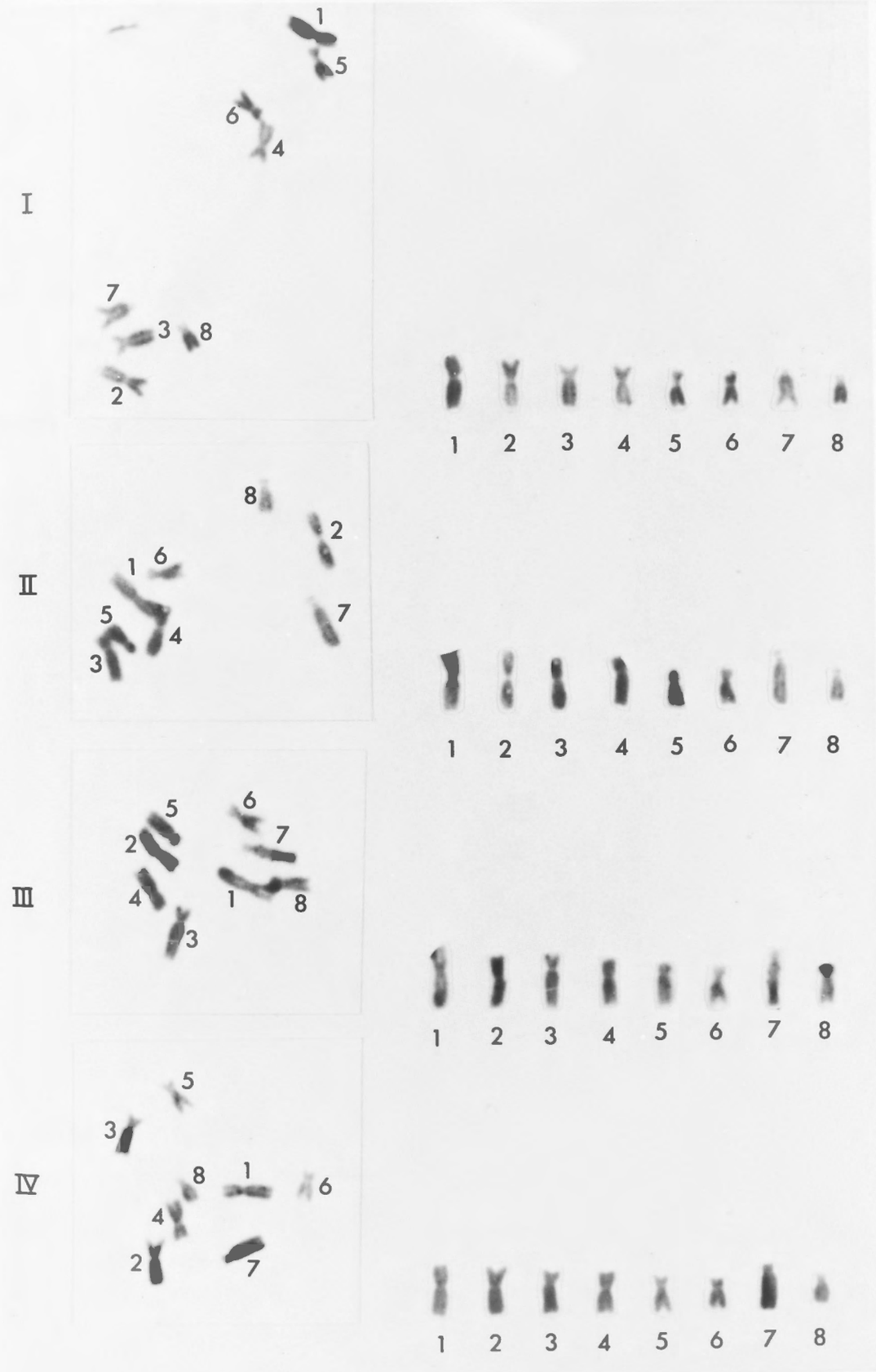
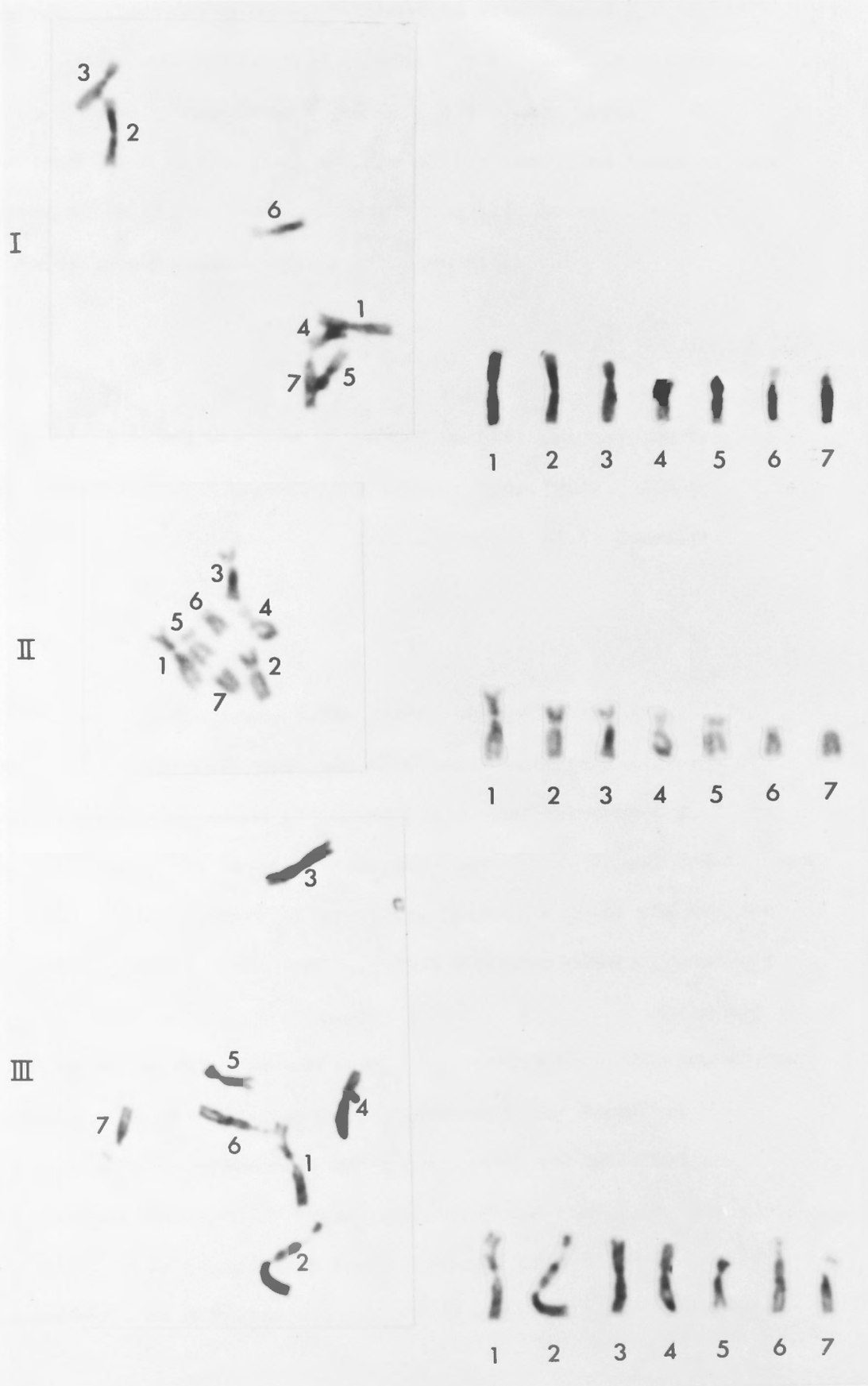

I
1
5
6
4
7
3
8
2
1 2 3 4 5 6 7 8
II
8
2
1
6
5
4
7
3
1 2 3 4 5 6 7 8
III
5
6
2
7
4
1
8
3
1 2 3 4 5 6 7 8
IV
5
3
8
1
6
4
2
7
1 2 3 4 5 6 7 8

FIG. 1.9 I → III show metaphase plates of 3 cells of the HA-1 culture, all having 7 chromosomes. The individual chromosomes show differences in gross morphology, as seen on the right-hand side of the figure.


I
3
2
6
1
4
7
5
1 2 3 4 5 6 7
II
3
6
5
4
1
2
7
1 2 3 4 5 6 7
3
III
5
7
4
6
1
2
1 2 3 4 5 6 7

chromosomes. Chromosomes 1, 2, 3 (and 4 in sets II and III) more closely resemble chromosome A of the diploid *H. gracilis* complement (see Fig. 1.1b). Chromosomes 6 and 7 of all 3 sets appear to be derived from the B chromosome, and the double satellite seems to have been retained on chromosome 6 of sets I and III, whilst a single satellite is seen on chromosome 7 of these sets.

C-banding

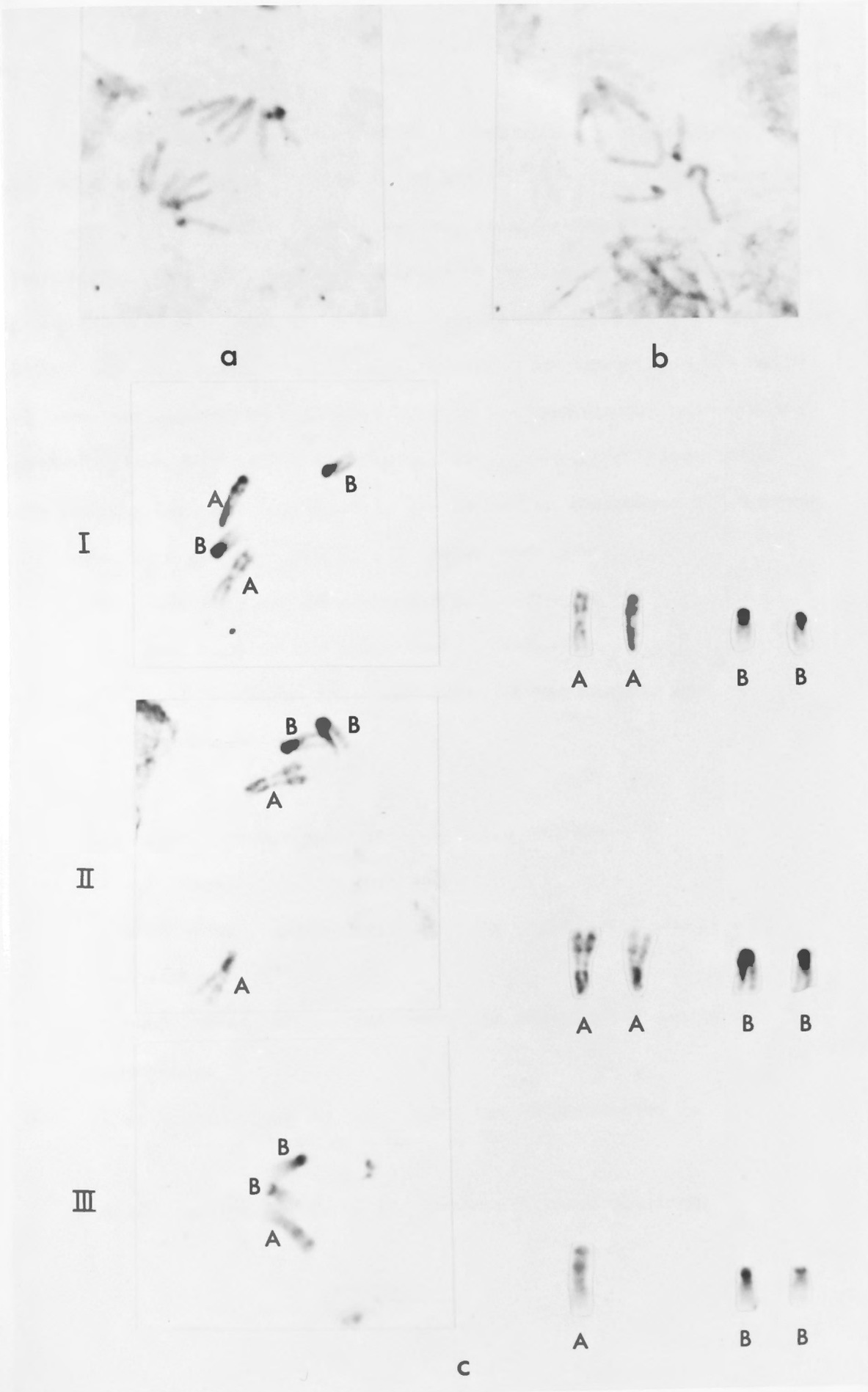
Giemsa C-banding has been attempted on this culture, to try and describe the 7 and 8 chromosome complements more fully. Comparison is made with the normal diploid root tip complement of *H. gracilis*.

Normal diploid:-

C-banded anaphase (Fig. 1.10a and b) and metaphase (Fig. 1.10c) complements of *H.gracilis* root tip show some consistent patterns of differential staining along the chromosomes. On chromosome A, 2 interstitial bands are observed, on the shorter arm, in all 3 metaphase cells in Fig. 1.10c. Other interstitial bands are seen, but are not consistently observed in all cells. Dark C-banded blocks are always seen on the short arm of chromosome B (Fig. 1.10 a → c). There may also be a band close to the centromere on the longer arm of this chromosome. Heteromorphism of the short arm of chromosome B may occur but is not obvious in these preparations. The C-band patterns described are similar to those observed by Tanaka and Taniguchi (1975) in this species. Further attempts to C-band this species should produce more clearly defined patterns of differential staining, as with *Crepis capillaris*.

FIG. 1.10 (a) and (b) show C-banded anaphase preparations from *Haplopappus gracilis* root tip.

(c) I → III show C-banded chromosomes from 3 different cells of *H. gracilis* root tip. Set III is incomplete.


a
b
I
A
B
B
A
A A B B
II
B B
A
A A B B
III
B
B
A
A B B
c

HA-1 culture:-

Four C-banded preparations of HA-1 suspension culture cells are presented in Fig. 1.11. Cells I, II and III have the 8 chromosome complement, as described in unbanded preparations (see Fig. 1.8). The chromosomes in cell IV are also assumed to be from an 8 chromosome set. This C-banding technique was not very successful on these cultured cells, mainly because the chromosomes were difficult to remove from the cells, and remained within the cell wall. Where the chromosomes were stained outside of the cell, as in IV of Fig. 1.11, the banding seems to be more clearly defined, particularly the blocks on chromosome 5. However, some bands were clearly seen in all cells, namely:-

(i) The dark band on the short arm of chromosome 8.

(ii) The dark band on the short arm of chromosome 7.

(iii) The band, close to the centromere, on the shorter arm of chromosome 5.

Other bands, which appear in some cells include:-

(i) A centromeric band on chromosome 1.

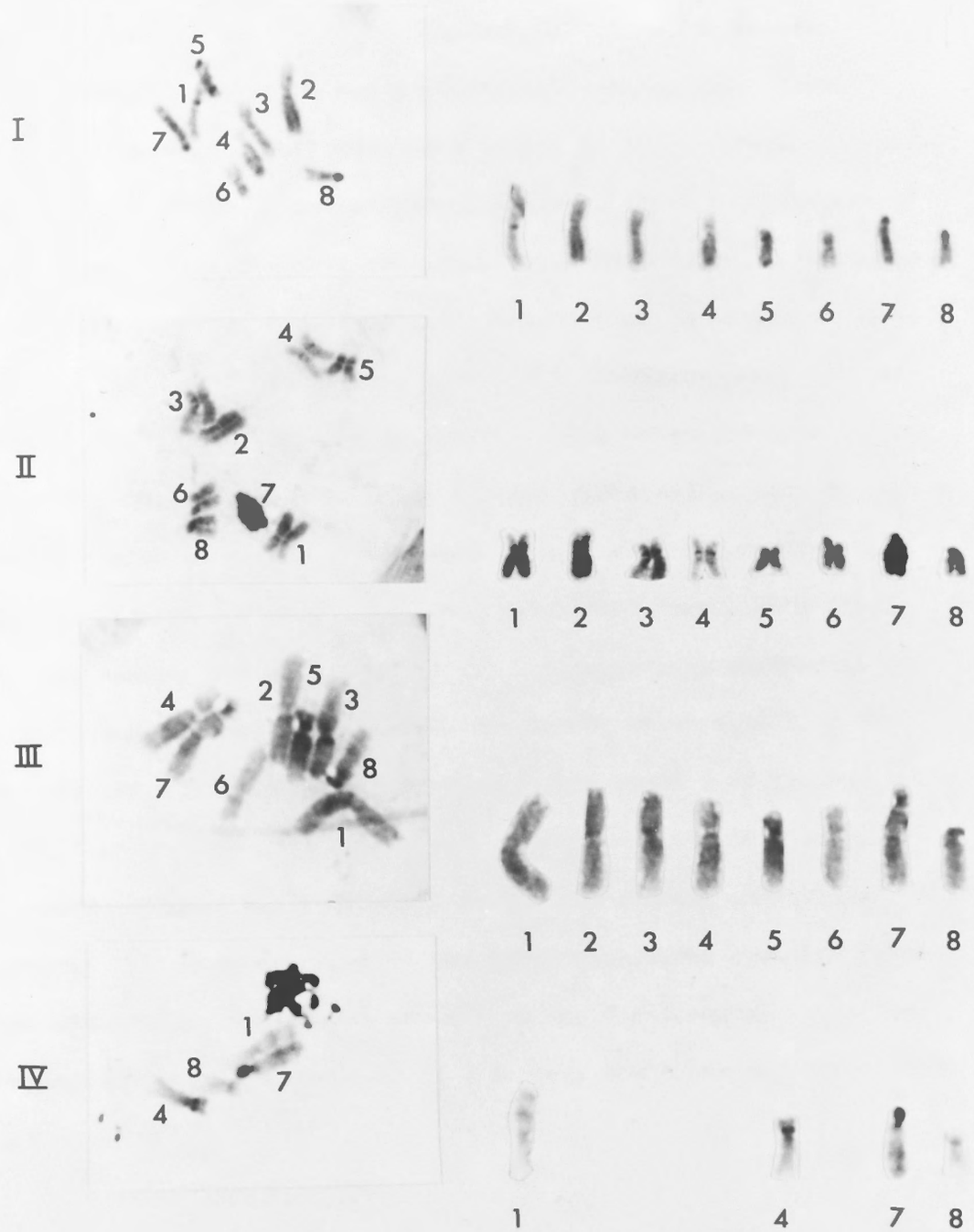
(ii) A band, close to the centromere, on the shorter arm of chromosome 4.

(iii) A band, close to the centromere, on the shorter arm of chromosome 3.

(iv) A telomeric block on the longer arm of chromosome 5.

No good C-banded 7-chromosome complements were obtained.

FIG. 1.11 Four C-banded metaphase plates of the HA-1 culture. Cells I → II have the 8 chromosome set, and cell IV seems to be part of such a set. Chromosomes seem to fall into 8 characteristic types.


I
5
1
3
2
7
4
6
8
1 2 3 4 5 6 7 8
II
4
5
3
2
6
7
8
1
1 2 3 4 5 6 7 8
III
4
2
5
3
7
6
8
1
1 2 3 4 5 6 7 8
IV
1
8
7
4
1 4 7 8

HA-3 suspension cell culture:-

This culture was initiated in February 1977 from the HA-new callus (see Table 0.1), which was predominantly tetraploid. After 4 months of culture a study of chromosome number in the population revealed that although the dividing cells predominantly retained an 8 chromosome complement, 18% of the dividing cell population had either a 7 chromosome or 6 chromosome set (see Fig. 1.12). Examples of all 3 karyotypes are shown in Fig. 1.13. It can be seen that the 8 chromosome karyotype is not a normal tetraploid complement, which would be composed of 4 large submetacentric chromosomes (the A chromosome) and 4 small, satellited, acrocentric chromosomes (the B chromosome), but, rather, consists of 5 larger chromosomes and only 3 smaller, satellited chromosomes (Fig. 1.13a). The 'extra' large chromosome may have arisen by an unequal translocation between an A chromosome (presumably chromosome 5 of the derived set) and a B chromosome (presumably chromosome 6 of the derived set). The 7 chromosome complement (Fig. 1.13b) consists of 5 larger submetacentric chromosomes and only 2 small, acrocentric satellited chromosomes. The heteromorphism of the short satellited arms is clearly observed, whilst this was not so obvious in the 8 chromosome set. The 6 chromosome karyotype is composed of 4 larger, and 2 smaller chromosomes (Fig. 1.13c).

Many abnormalities of mitosis were observed in this culture, at this time, including chromosome bridges, lagging chromosomes at anaphase, and micronuclei (Fig. 1.14).

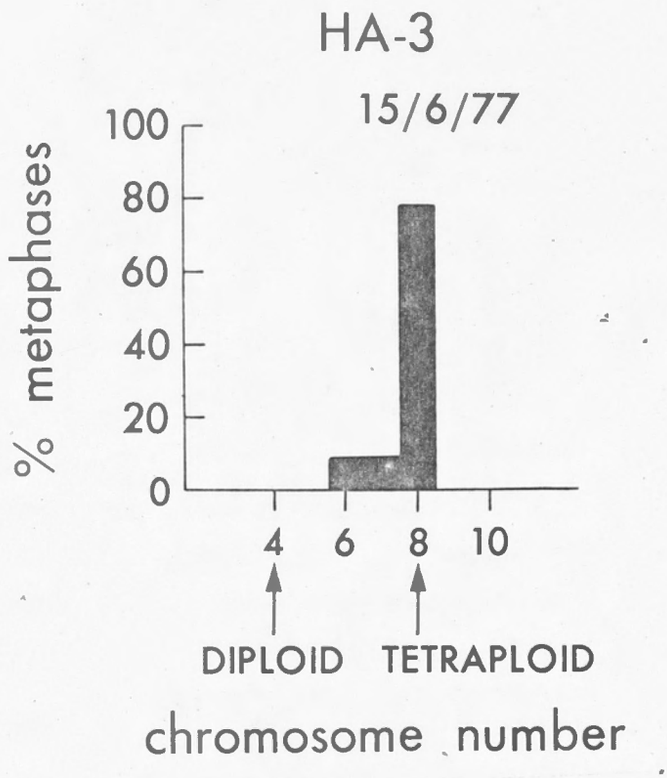


FIG. 1.12 Frequency distribution of chromosome numbers observed in 40 dividing cells of the HA-3 suspension cell culture on 15/6/77.

FIG. 1.13 Orcein stained preparations of metaphase cells of the HA-3 culture, showing (a) 8 chromosome set, (b) 7 chromosome set, (c) 6 chromosome set. The possible pathway of chromosome reduction is indicated.

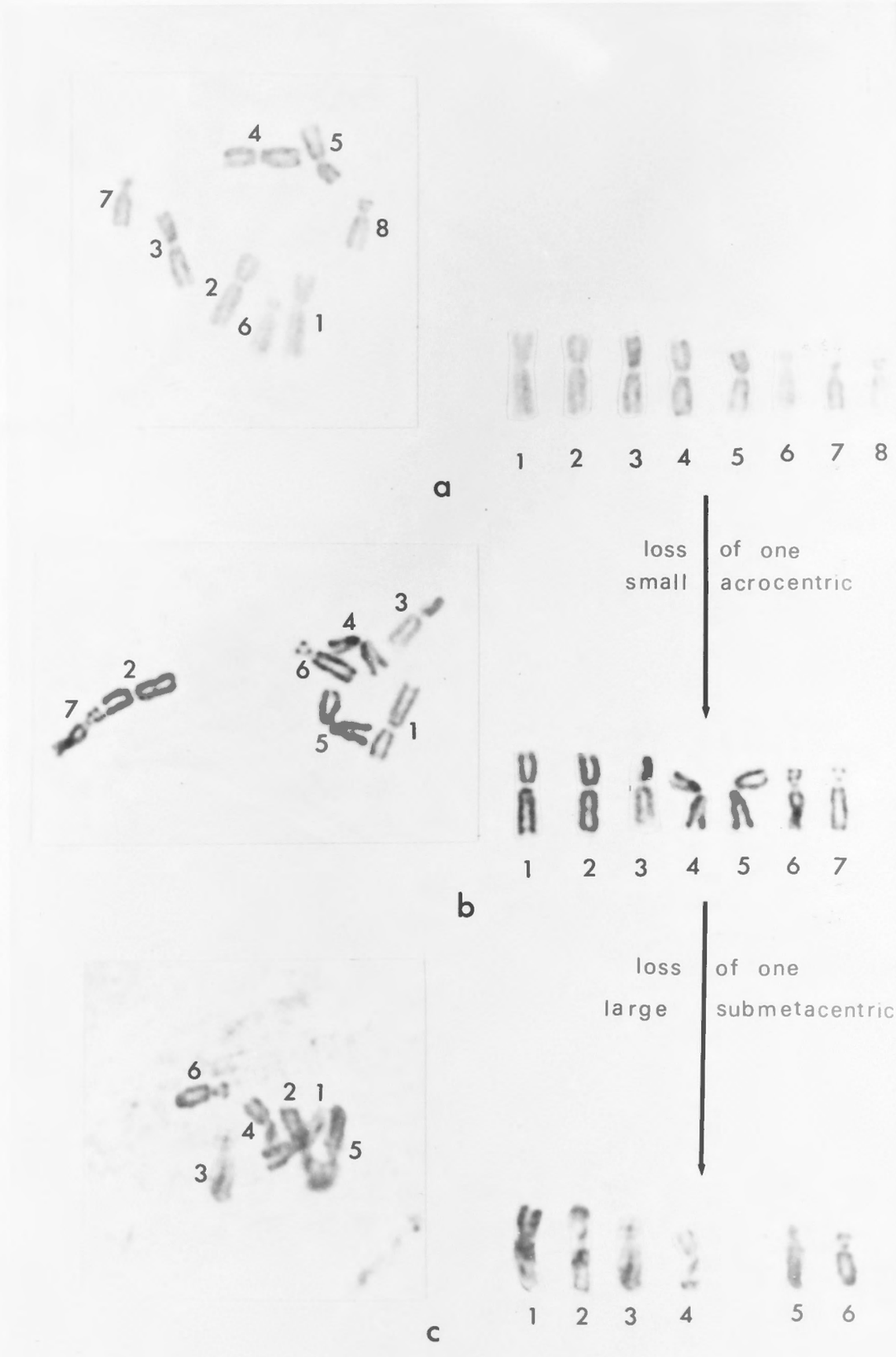
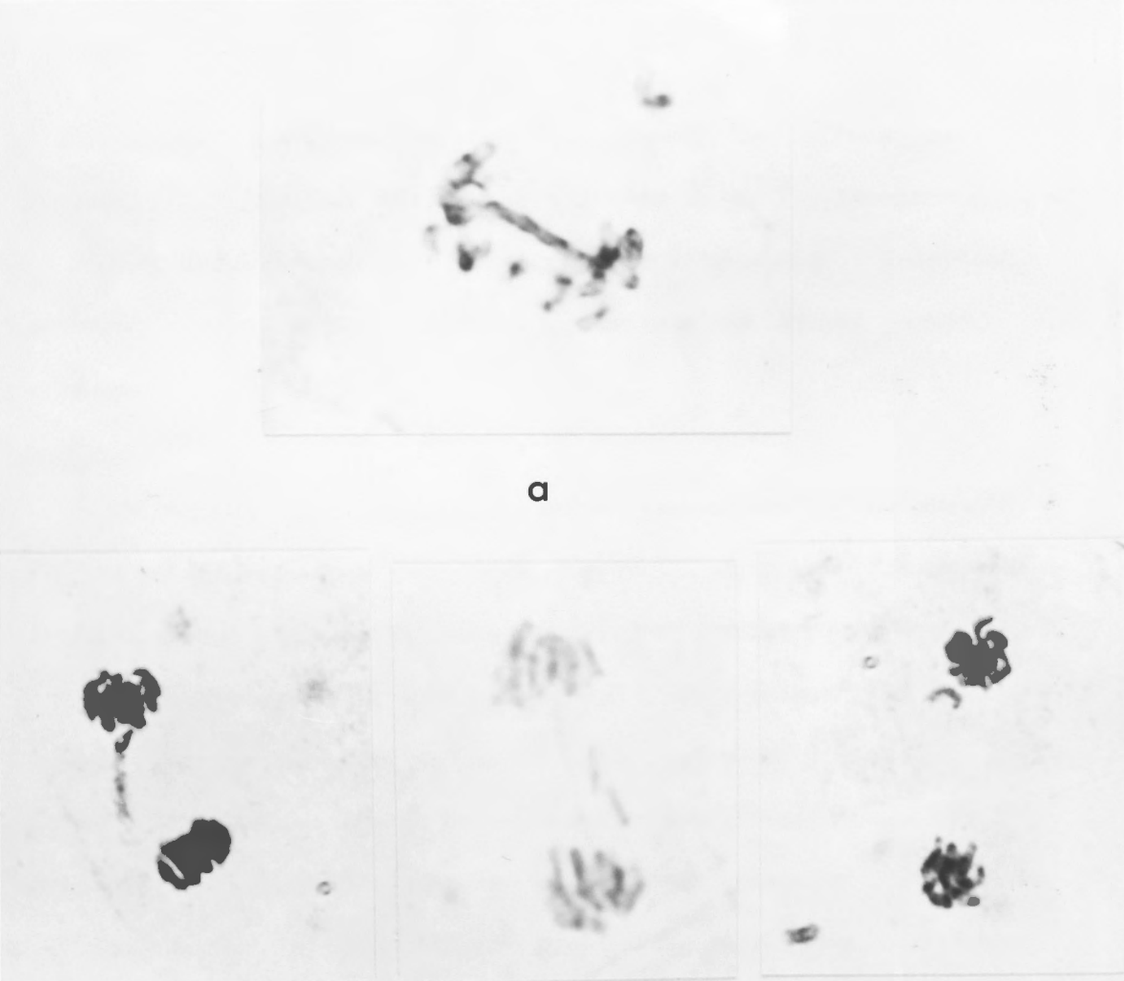

4
5
7
8
3
2
6
1
1 2 3 4 5 6 7 8
a
loss of one small acrocentric
3
4
2
6
7
1
5
1 2 3 4 5 6 7
b
loss of one large submetacentric
6
2 1
4
5
3
1 2 3 4 5 6
c

FIG. 1.14 Abnormalities of division in the HA-3 culture.
(a) chromosome bridge, (b) lagging chromosomes at anaphase,
(c) micronuclei.



a

b

c

1.3.2.3 *Brachycome dichromosomatica*

Lin_{31} and Lin_{113}:-

Chromosomal monitoring of these cell suspension cultures was performed by A.R. Gould. Whilst the cultures initially remained diploid for 2 years, a few tetraploids were seen after this time. Aneuploid karyotypes, comprising 7 and 5 chromosomes have now arisen in the population.

Following the introduction of banding techniques by the present author, some attempts have been made, by Gould, to C-band *Brachycome dichromosomatica*. Fig. 1.15 shows the banded complement in the root tip of a plant produced in a cross between plants raised from seed collected from the field in 1977. Each chromosome of the diploid complement can be identified due to heteromorphic satellites on the small chromosomes and presence or absence of a large C-positive block on the large chromosome. In line with the present authors' study, Gould has initiated tissue cultures from this plant for chromosome instability studies.

1.4 DISCUSSION

1.4.1 *Crepis capillaris*

Whilst the original CAPT callus was predominantly diploid, the suspension cell culture derived from this callus gained a hyperdiploid karyotype, consisting of 7 chromosomes, after just 1 year of culturing with regular 7 day transfers (Fig. 1.2). Prior to the complete dominance, in the suspension cell culture, of a 7 chromosome complement, some tetra-

FIG. 1.15 C-banded complement of the root tip of a plant produced by a cross of 2 plants of *Brachycome dichromosomatica*.

ploidy was observed, indicating that chromosome doubling may have been a first step in karyotypic evolution of this cell culture.

C-banding analysis of the normal, diploid, root tip of *Crepis capillaris*, and of the CAPT culture, has allowed comparison of the 2 cell populations, and exploration of possible mechanisms for the derivation of the CAPT genome from the original diploid.

In this report, the patterns of C-banding along metaphase chromosomes of *Crepis capillaris* root tip are more clearly defined than in the two previous studies of this species (Schweizer, 1973; Tanaka and Komatsu, 1977). Additionally, both prior attempts failed to reveal as many C-banded regions as presently shown, although those bands which were reported by Tanaka and Komatsu have all been seen in the present study.

C-banding in the CAPT culture has revealed that the dominant, 7 chromosome complement of this culture is the same in all dividing cells. Such dominance of a particular karyotype commonly occurs in plant cell cultures (Bayliss and Gould, 1974; Singh, 1975), presumably because cells containing a specific chromosome complement are better suited to survival in the specialised environment of a tissue culture system. In the case of this CAPT culture, the cell cycle in the dividing population is extremely short in duration, and the G_1 phase is virtually non-existent (Ashmore and Gould, 1979; see also 2nd chapter of this thesis). Thus, cells with the 7 chromosome complement observed in this cell line, may have dominated because of their capacity for such rapid cell cycle traverse. Certainly, it is known that changes in chromosome constitution can cause alterations in cell cycle timing (Krune and

Wolf, 1977). The proportion of heterochromatin in the chromosome complement may also affect cell division rate (Barlow, 1973), so that the increased heterochromatin content of the CAPT cells may have affected the ability for survival in a cell population initially containing mixed karyotypes.

Recently, there has been some doubt expressed about the stem cell concept of animal tumours (Hashmi *et al.*, 1974), since, in the original studies, the most reliable criterion of the stem cell was chromosome number. In the case of the CAPT culture, however, careful observation has revealed a single C-banded karyotype, but further investigation would be necessary to reveal whether this apparent adherence to the stem line concept in the CAPT culture is a common characteristics of all plant tumours.

The hyperdiploid, 7 chromosome karyotype of the CAPT cell line deviates considerably from the normal diploid complement of *Crepis capillaris* (see Fig. 1.6). Bayliss (1980), has recently reviewed the available literature on chromosomal instability in plant tissue cultures. He comments that in nearly all of the 55 different plant species studied there is a deviation from the conventional karyotype of the cells of the intact plant. The abnormal karyotype observed in the CAPT culture is therefore consistent with these findings. A comparison of the CAPT chromosome complement with that of the normal diploid karyotype indicates that not only is there an extra chromosome present in the CAPT genome, but, additionally, all chromosomes differ in length from the original root tip chromosomes and have strikingly different C-band patterns, suggesting that numerous structural chromosomal

rearrangements have occurred during the formation of the CAPT chromosome complement. Although chromosomal rearrangements are certainly observed in some plant tissue cultures (Bayliss, 1980), and, particularly, in *Crepis capillaris* cell lines (Sacristán and Wendt-Gallitelli, 1973), such extensive rearrangements have not previously been indicated. It may be that such major chromosomal change is associated with the tumorous nature of the CAPT cell line, and that the integration of the Ti plasmid into the plant genome may possibly affect chromosomal stability. Alternatively, a similar degree of structural change may, in fact, also occur in other plant cell lines, but has not been revealed by previous studies, since banding techniques have not been used.

From a comparison of the C-band patterns of the CAPT genome with that of the root tip, it is possible to make certain suggestions about the derivation of the CAPT genome. Proposed schemes for the complete derivation of chromosomes 7, 6 and 4 are as follows:-

(i) Chromosome 7 is most likely derived from the SAT chromosome as represented diagrammatically in Fig. 1.16 a. This involves breakage of the SAT chromosome between the centromere and the C-positive satellite blocks, i.e. at the secondary constriction. Although no short arm is visible on chromosome 7, this may in fact be present, but difficult to detect, since the DNA at the site of a secondary constriction is highly despiralized. A considerable deletion of the long arm of chromosome SAT must also have occurred.

(ii) Chromosome 6 also appears to be derived from the SAT chromosome by a similar mechanism (see Fig. 1.16 b). However, in this case, there must be smaller deletion of the long arm of SAT.

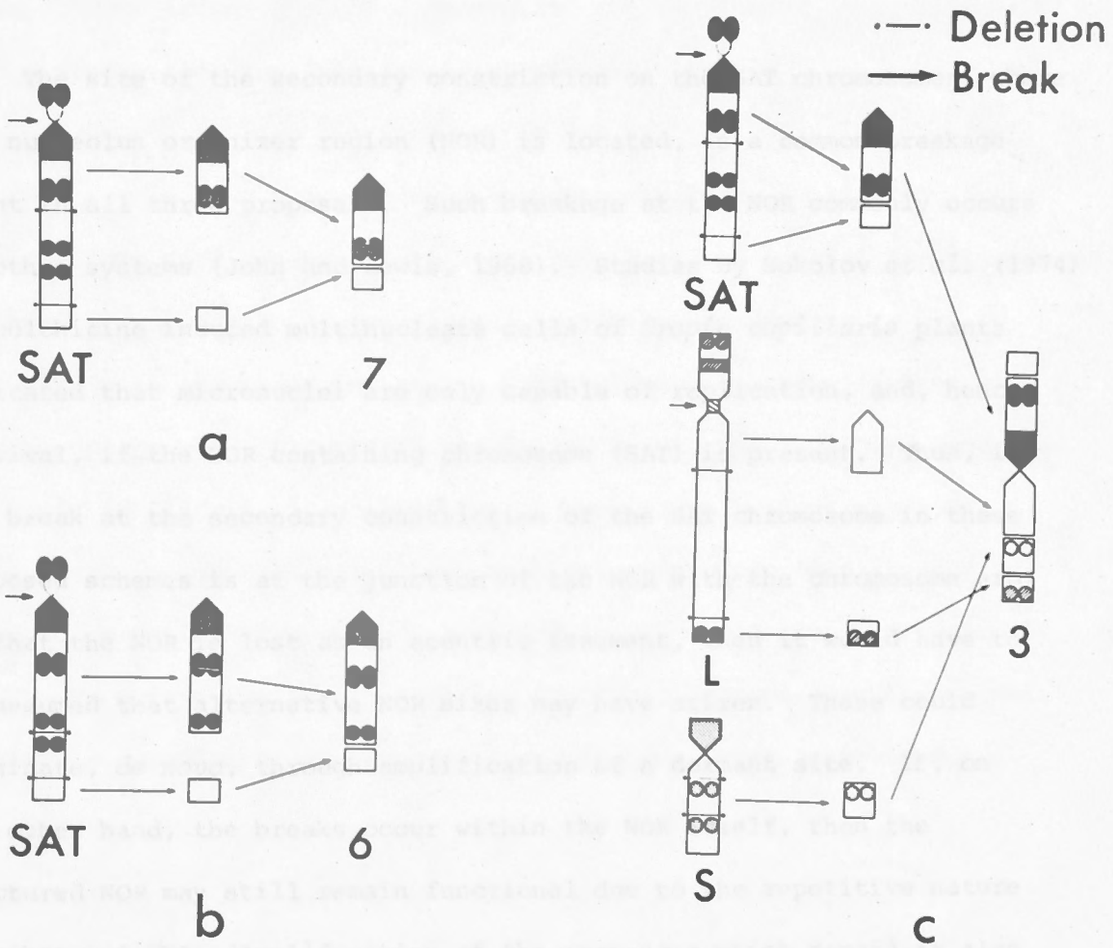


FIG. 1.16 (a) Scheme for the derivation of chromosome 7 of the CAPT genome from the SAT chromosome of the root tip.

(b) Scheme for the derivation of chromosome 6 of the CAPT genome from the SAT chromosome of the root tip.

(c) Scheme for the derivation of chromosome 3 of the CAPT genome, involving all 3 chromosome types (L, SAT and S) of the root tip.

(iii) The slightly shorter arm of chromosome 3 of the CAPT genome may have been derived from the long arm of the SAT chromosome. Again, this would involve breakage at the secondary constriction, together with a deletion in the long arm. The longer arm of chromosome 3 may have arisen from chromosomes L and S, of the root tip complement, as set out in Fig. 1.16 c.

The site of the secondary constriction on the SAT chromosomes, where the nucleolus organizer region (NOR) is located, is a common breakage point in all three proposals. Such breakage at the NOR commonly occurs in other systems (John and Lewis, 1968). Studies by Sokolov *et al.* (1974) on colchicine induced multinucleate cells of *Crepis capillaris* plants indicated that micronuclei are only capable of replication, and, hence, survival, if the NOR containing chromosome (SAT) is present. Thus, if the break at the secondary constriction of the SAT chromosome in these proposed schemes is at the junction of the NOR with the chromosome arm, so that the NOR is lost as an acentric fragment, then it would have to be assumed that alternative NOR sites may have arisen. These could originate, *de novo*, through amplification of a dormant site. If, on the other hand, the breaks occur within the NOR itself, then the fractured NOR may still remain functional due to the repetitive nature of ribosomal DNA. Amplification of the sequences which remain is also possible. The application of techniques which cytologically stain NOR regions would obviously be of value to examine these possibilities.

It is also possible to propose explanations concerning the origin of parts of the other four chromosomes of the CAPT genome. The long arm of chromosome 2 has most likely originated from the long arm of

chromosome L, since both the telomeric and centromeric C-blocks of that chromosome are present. The spacing of the double C-band blocks on chromosomes 1 and 2 of the CAPT genome suggest that these may originate from the long arm of the SAT chromosome. Similar blocks also appear on chromosomes 4 and 5, with increased material in the lower block. It is also possible that these double blocks of C-positive material have arisen through differential replication of other regions of the genome *e.g.* the long arm of chromosome S.

Since more than two copies of chromosome SAT are implicated in the origin of the CAPT genome, there would have to be an initial evolution in the CAPT culture to a hyperdiploid karyotype, presumably to tetraploidy in the first instance. Polyploidisation certainly occurs in other plant cell cultures (Bayliss, 1980), and has also been reported in *Crepis capillaris* tissue cultures by many authors (Bayliss, 1980). It is worth noting that polyploidy must arise *in vitro* in this species, since no polysomy has ever been observed in the whole plant (Brossard, 1978). To obtain the abnormal aneuploid CAPT karyotype from the normal tetraploid complement, chromosomal loss, combined with rearrangements, must occur.

Aneuploidy may arise in tissue cultures by errors of division in polyploid cells. The large, vacuolated nature of plant cells in culture may be a cause of difficulties in the division process. Mitotic abnormalities (including lagging chromosomes at anaphase, chromosome bridges, multipolar spindles), are observed in plant tissue cultures (Bayliss, 1973), and it is believed that such abnormalities are more readily tolerated by polyploid cells, since loss of chromatin leads to

less genic imbalance than loss from a diploid cell. In *Crepis capillaris*, Sidorov and Sokolov (1963), have shown the appearance of up to 25% aneuploidy in plants following the induction of tetraploidy by colchicine treatment. Sacristán (1971), also observed progressive loss of chromosomes from a tetraploid clone of *Crepis capillaris* callus cells, to produce a hyperdiploid karyotype.

Chromosomal rearrangements are also known to occur more readily in polyploidised cells in culture than in diploid cells in both plant (Sacristán, 1971) and animal systems (Hsu, 1961; Halfer *et al.*, 1980). All types of known chromosomal change (deletions, inversions, duplications, translocations) are detectable in polyploid *Drosophila* cell lines (Dolfini and Halfer, 1978). Chromosomal change by translocation has previously been observed in *Crepis capillaris* (Sacristán and Wendt-Gallitelli, 1973), and both translocations and inversions have been found in C-banded cells of *Vicia faba* callus (Papeš *et al.*, 1978). It is worth noting than an increase in heterochromatin content has been found to follow chromosomal rearrangement in cell cultures (Halfer *et al.*, 1980), and such an increase is also known from studies in natural populations *e.g.* in some Australian frog species of the genus *Litoria*, in which King (1980) has proposed that "extra" heterochromatin has arisen both by amplification and by euchromatin transformation.

Thus, the proposed scheme for karyotype evolution, of tetraploidisation followed by segregation and rearrangement, has substantial support from similar studies in both plant and animal populations. Such a scheme was proposed by Bayliss and Gould (1974) to explain the origin of aneuploidy in *Acer pseudoplatanus* suspension cell cultures, and is

also a common pathway of karyotype evolution in both normal and tumorous animal cells in culture (Levan and Biesele, 1958; Hsu, 1961; Terzi and Hawkins, 1975), and in *Drosophila* cell lines (Dolfini and Halfer, 1978).

By one simple change it is possible, on the other hand, to produce a 6 chromosome karyotype from the 7 chromosome karyotype observed in CAPT cells. This change involves fusion, at the centromeres, of chromosomes 6 and 7 of the CAPT genome. It can be observed that there are similarities in C-banded blocks between chromosomes 1 and 2, and chromosomes 4 and 5 of the CAPT genome. Additionally, chromosomes 3, 6 and 7 have similarities, having all probably originated from the SAT chromosome of the whole plant. It is possible, then, to arrange the 7 CAPT chromosomes into a 6 chromosome set of 3 chromosome 'pairs', chromosome 3 being paired with 6 and 7 combined (see Fig. 1.17 b).

It is possible to suggest mechanisms by which the 6 chromosome 'pairs' depicted in Fig. 1.17b have originated from the basic diploid *Crepis capillaris* complement, as follows:-

(i) Chromosome 3 and chromsome 6/7 combined may have derived from the SAT chromosome by the mechanism set out in Fig. 1.18a. This involves a pericentric inversion and despiralization of the secondary constriction. Loss of the procentric C-band block, plus a paracentric inversion in the long arm gives chromosome 3. Absence of such a procentric C-band block occurs in the neo Y of *Stenacatantops augustifrons*, a species of Acridoid grasshopper, compared to its presence in the homologous arm of the neo X (King and John, 1980). To produce chromosome 6/7 some amplification of unbanded material must occur.

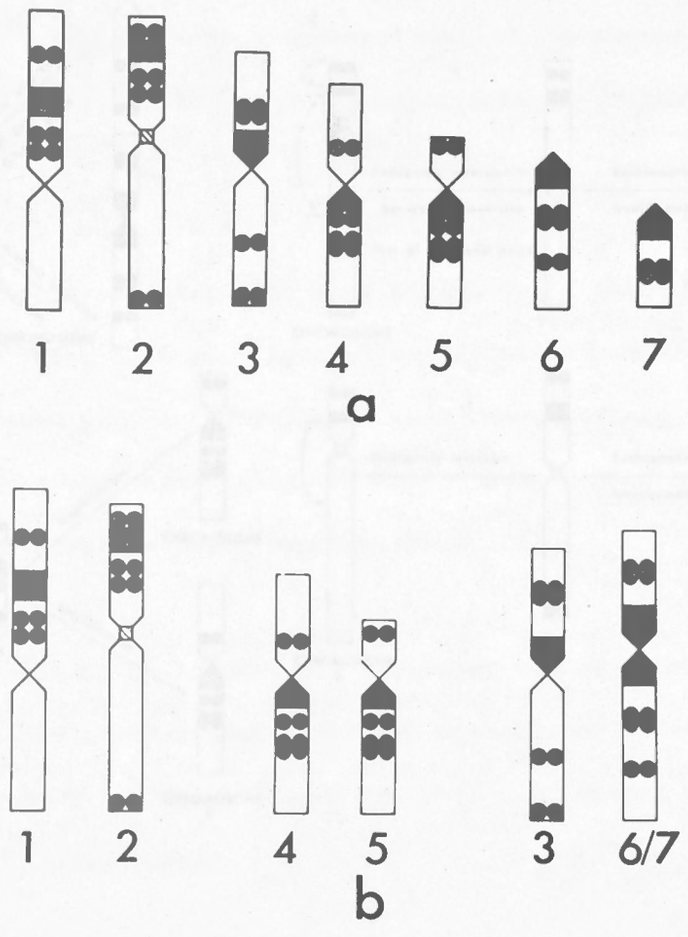


FIG. 1.17 (a) Idiogram of the C-banded karyotype of the CAPT cell line.

(b) Idiogram of the 3 proposed "pairs" of chromosomes which may be derived from the CAPT karyotype by a simple fusion of chromosomes 6 and 7.

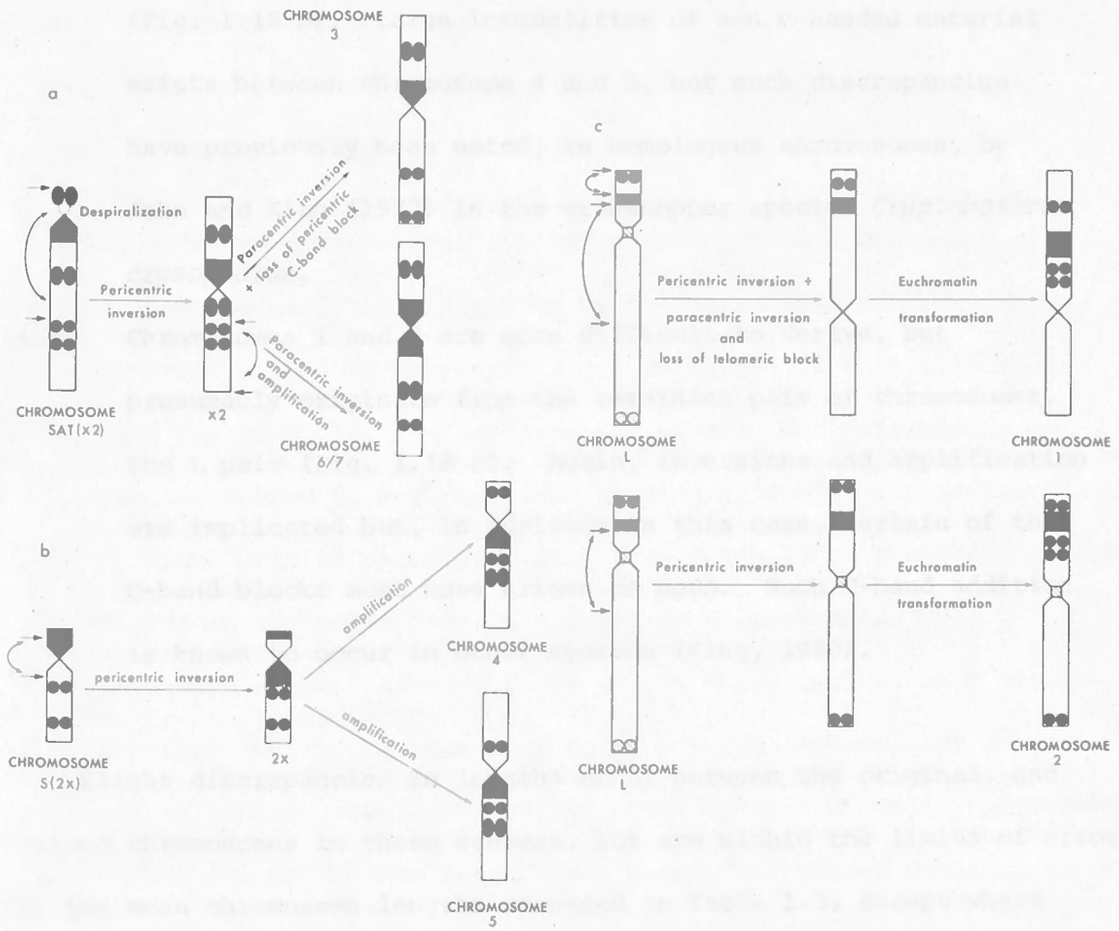


FIG. 1.18 (a) Scheme for the derivations of chromosomes 3 and 6/7 combined of the CAPT genome from the SAT chromosome of the root tip.

(b) Scheme for the derivations of chromosomes 4 and 5 of the CAPT genome from chromosome S of the root tip.

(c) Scheme for the derivations of chromosomes 1 and 2 of the CAPT genome, each from chromosome L of the root tip.

(ii) Chromosomes 4 and 5 may be derived from the S chromosome, by a simple pericentric inversion, followed by considerable amplification of chromatin, particularly in chromosome 5 (Fig. 1.18 b). Large inequalities of non C-banded material exists between chromosome 4 and 5, but such discrepancies have previously been noted, in homologous chromosomes, by John and King (1977) in the grasshopper species *Cryptobothrus crysophorus*.

(iii) Chromosomes 1 and 2 are more difficult to derive, but presumably originate from the remaining pair of chromosomes, the L pair (Fig. 1.18 c). Again, inversions and amplification are implicated but, in addition in this case, certain of the C-band blocks must have arisen *de novo*. Such C-band addition is known to occur in other systems (King, 1980).

Slight discrepancies in lengths occur between the original, and derived chromosomes in these schemes, but are within the limits of error for the mean chromosome lengths recorded in Table 1.3, except where amplification has been indicated.

This scheme of karyotypic change proposes initial derivation of the 6 chromosome complement (Fig. 1.17 b) by the mechanisms outlined above, and represented diagrammatically in Fig. 1.18. The observed 7 chromosome karyotype of the CAPT culture would then be obtained from this 6 chromosome set by a simple dissociation at the centromere of chromosome 6/7. Such a system of karyotypic evolution, by-passing a polyploid step, would be unusual as compared with other reports concerning plant tissue cultures, and may be associated with the tumorous state of this plant

tissue culture. However, such complex karyotypic change is less likely to have occurred within a diploid genome, so that the preferred scheme for derivation of the CAPT karyotype from the normal diploid is that which involves initial polyploidy.

1.4.2 *Haplopappus gracilis*

The pathway of karyotypic evolution in the HA-1 suspension cell culture seems to have been initiated by the appearance of tetraploidy. A 6 chromosome aneuploid karyotype later dominated in this culture throughout the time that a 4 day transfer program was retained. Following the change to a 7 day transfer program karyotypic alteration again occurred with 12 chromosome sets plus a few 11 or 8 chromosome sets arising in the population, followed, at a later data, by a 7 chromosome complement. After 2 years of the 7 day regime, an 8 chromosome karyotype (which was found not to be a normal tetraploid) formed the major chromosome complement in the culture, with a smaller percentage (25%) having a 7 chromosome set. Thus, again, chromosome doubling is implicated in the initial stages of evolution towards a dominant karyotype.

It can be suggested then that, as with the CAPT culture, a scheme for karyotypic evolution involves initial chromosome doubling followed by chromosome loss and rearrangements. Chromosomal rearrangements are certainly included in this scheme, since the final 8 and 7 chromosome complements both show deviations from the basic diploid chromosome types. It is not known whether the 6 chromosome set, which previously dominated the culture, included structural chromosome change. Singh (1975), previously observed a 6 chromosome karyotype as the major complement in

a suspension cell culture and in this case one obvious rearrangement was noted. Several authors have supported the view that evolution of the karyotype towards aneuploidy in *H. gracilis* cell culture begins with tetraploidisation (Shamina, 1972; Singh, 1975; Singh and Harvey, 1975) and that this chromosome doubling occurs *in vitro* rather than arising from polyploid cells in the original explant. This view is supported by the appearance of 12 chromosome sets in an HA-1 culture which was previously dominated by a 6 chromosome karyotype.

The fact that karyotypic change, from the 6 chromosome set, occurred in the culture following the change from a 4 day to a 7 day transfer regime is not surprising. Even slight alterations in culture conditions, such as altered innoculum volume may cause a directional change in chromosome constitution (Kaziwara, 1954). Also, it has been noticed in *Haplopappus gracilis* (Singh and Harvey, 1975), and *Daucus carota* (Bayliss, 1975a) cell cultures that a prolongation of stationary phase causes the increased appearance of tetraploids. The change to a 7 day culture period may then be responsible for the shift towards chromosome doubling.

C-banding analysis, on the 8 chromosome set in the final culture is summarised in Fig. 1.19, along with proposed derivations of the chromosomes from the original A and B chromosomes of the toot tip. Both chromosomes 7 and 8 of this complement are derived from chromosome B, indicated by the darkly staining short arm. In addition, the C-band block on the short arm of chromosome 5 indicates that this chromosome is also derived from the B chromosome. Chromosome 6 does not show this C-block so clearly, but this may be due to problems with the banding

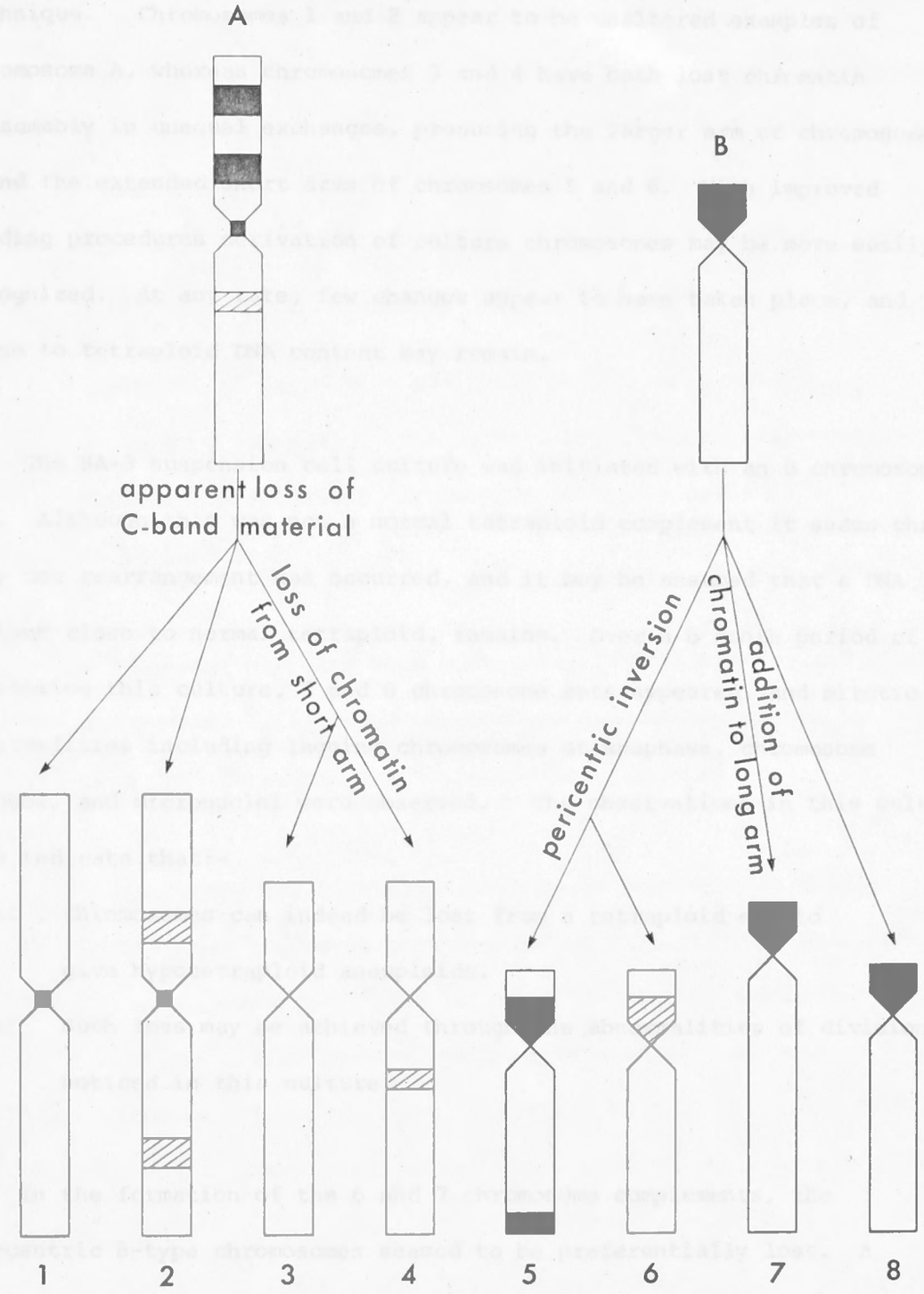


FIG. 1.19 Schemes for the likely origin of each chromosome of the 8 chromosome set of HA-1 from the 2 chromosome types of *H.gracilis* root tip (*i.e.* normal diploid).

technique. Chromosomes 1 and 2 appear to be unaltered examples of chromosome A, whereas chromosomes 3 and 4 have both lost chromatin presumably in unequal exchanges, producing the larger arm of chromosome 1 and the extended short arms of chromsomes 5 and 6. With improved banding procedures derivation of culture chromosomes may be more easily recognized. At any rate, few changes appear to have taken place, and close to tetraploid DNA content may remain.

The HA-3 suspension cell culture was initiated with an 8 chromosome set. Although this was not a normal tetraploid complement it seems that only one rearrangement has occurred, and it may be assumed that a DNA content close to normal tetraploid, remains. Over a 6 month period of monitoring this culture, 7 and 6 chromosome sets appeared, and mitotic abnormalities including lagging chromosomes at anaphase, chromosome bridges, and micronuclei were observed. The observations in this culture thus indicate that:-

(i) Chromosomes can indeed be lost from a tetraploid set to give hypotetraploid aneuploids.

(ii) Such loss may be achieved through the abnormalities of division noticed in this culture.

In the formation of the 6 and 7 chromosome complements, the acrocentric B-type chromosomes seemed to be preferentially lost. A similar observation was made in *H. gracilis* (Singh and Harvey, 1975) and *Vicia hajastana* (Singh *et al.*, 1972) cell cultures, where the largest chromosome was represented most frequently in aneuploid karyotypes. However, it appears that 2 of the satellited chromosomes are always retained in the 6 and 7 chromosome sets of HA-3, and this was also the

case with the 7 and 8 chromosome complements of the HA-1 culture. It is known that the NOR region is on the small arm of the B chromosome, so that this region is being retained, in its original form, and number in both these cultures.

1.4.3 *Brachycome dichromosomatica*

The patterns of karyotypic evolution in the Lin_{31} and Lin_{113} suspension cell cultures also seem to have involved initial tetraploidisation followed by a loss of chromosomes to give a hyperdiploid chromosome complement.

Some C-banding has also been successful in this species and in the example shown (Fig. 1.15) all 4 chromosomes are different, making this plant a good candidate for genetic manipulation studies.

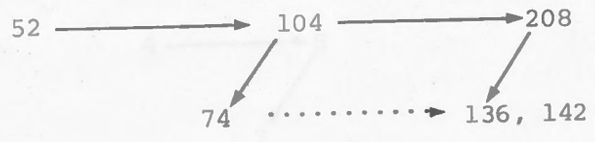
Preliminary studies on tissue cultures of this species (Gould, 1978; Gould, 1979a) suggested that chromosome stability was maintained for long periods. However, first tetraploids and then aneuploids appeared when the callus cultures were introduced to a suspension culture environment (Gould, personal communication). C-banding studies should clarify the pathways of chromosomal evolution in cultures of this species.

1.4.4 General Discussion

By using these 3 composites with extremely low chromosome number, it has been possible to accurately monitor the chromosomal characteristics of the derived cell cultures of these species. By the additional use of

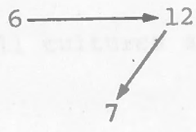
the Giemsa C-banding technique which has previously only been used, somewhat unsuccessfully, in 2 other tissue culture systems (Papeš *et al.*, 1978; Wochok *et al.*, 1980) it has also been possible to indicate the nature of any chromosomal rearrangements occurring. This has been particularly successful with the CAPT culture where C-banded patterns were very clear, and the rearrangements were very extensive. The banding in *H. gracilis* was less successful but, with the removal of the chromosomes from the cells, more clearly defined banding patterns should be obtained.

Deviation from the original diploid karyotype in all 3 species occurs in suspension cell culture, and a common pattern of karyotype change has emerged. This involves chromosome doubling and subsequent chromosome loss from the tetraploid set to give rise to a stable, dominant aeuploid. It should be noted here that the 8 chromosome complement of the final HA-1 culture may be considered as an 'aneuploid' since it probably arose from a 12 chromosome set. Previously line diagrams could be used to illustrate the course of karyotypic evolution in plant tissue cultures *i.e.* for *Acer pseudoplatanus* (Gould, 1975), the pattern of chromosomal change was:-



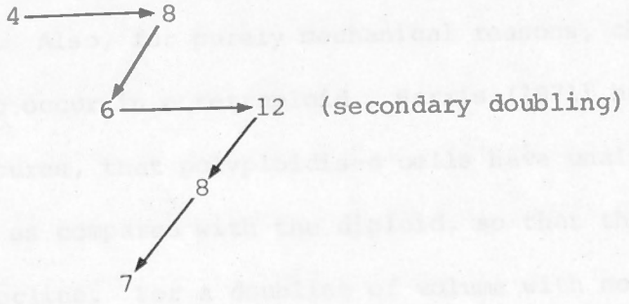
The chromosome numbers are however, only modal numbers since accurate counting was not possible. Such diagrams can be constructed for the 3 composite species, as follows:-

C. Capillaris (CAPT)



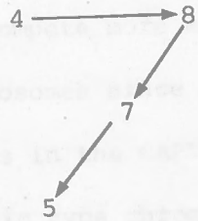
H. gracilis

(1) HA-1



(2) HA-3

B. dichromosomatica Lin_{31} and Lin_{113}



C-banding analysis of both CAPT and HA-1 has revealed that chromosome rearrangements occur, whereas these diagrams do not include this information, but merely relate to centromere number in the cultures.

As mentioned previously, a general pattern of tetraploidisation followed by loss to give aneuploidy is a common pathway of chromosomal evolution in animal cell cultures and other plant systems.

It is suggested that the aneuploid complement arises from a tetraploid set since extensive chromosomal change is more likely to be tolerated by a polyploid cell as this leads to less genic imbalance than in a diploid cell. Also, for purely mechanical reasons, chromosomal loss may be expected to occur in a tetraploid. Harris (1971) has shown, in pig kidney cell cultures, that polyploidised cells have unaltered dimensional properties as compared with the diploid, so that the surface to volume ratio must decline. For a doubling of volume with no change in proportions the expected increase in the area per cell is only 1.59 times. It may be that the spindle apparatus of the tetraploid cell is unable to cope with the doubled chromosome number, given that the spindle plate area may only be increased by a factor of 1.59. Thus, competition for attachment of centromeres to the spindle at metaphase may occur, leading to the exclusion of some chromosomes from the telophase nuclei. Terzi (1972) has noticed a positive selection for cells with a diploid centromere number. Added to this is the suggestion that metacentric type chromosomes may compete more effectively for spindle space than acrocentric type chromosomes since there is an increased proportion of metacentric chromosomes in the CAPT and HA-1 genomes. Preferential retention of metacentric type chromosomes has previously been observed in tumorous *Crepis capillaris* (Sacristán and Wendt-Gallitelli, 1973), and in some animal tumours (Hsu, 1959; Muldal *et al.*, 1971). It may be that there is an unbalanced drag on acrocentric chromosomes which could cause their preferential loss at anaphase. It has generally been

observed that small chromosomes function at anaphase with fewer kinetochore microtobules (kMT's) than large chromosomes (Fuge, 1978) and also Moens (1979) has noticed that chromosomes which form as a result of Robertsonian fusion initiate a far larger number of MT's than telocentrics. Such differences in numbers of kMT's could create a situation where large metacentric chromosomes move more effectively on the spindle and are therefore preferentially retained in a competitive situation.

It would however be naive to suggest that the tetraploidisation-segregation pattern is the only interpretation of the results presented for these 3 species. As noted for the CAPT culture it is possible, though less likely, that rearrangements may have occurred within the diploid set.

As for the use of these 3 species for genetic manipulation studies it seems that once the cultures have reached the stage of having a stable, aneuploid karyotype, they may be suitable for such experiments. However, it may be important to maintain a strict adherance to a culture regime in order to prevent further karyotypic change. In addition it is obviously important to carefully analyse the derived chromosome complement, preferably with banding techniques since karyotypes which at first sight appear to be normal diploids or tetraploids may in fact include several structural chromosome alterations. Careful analysis of chromosomes by C-banding can also be useful for later identification in hybrid situations.

An alternative approach, and one more commonly considered, is to try and maintain diploidy in the cultures for use in such manipulation

experiments by alteration of culture regime *e.g.* a shorter stationary phase may allow diploids to compete more successfully. Other workers have advocated the use of parafluorophenyl alanine (PFP) (Gupta and Carlson, 1972) to preferentially favour the proliferation of diploids. This approach views chromosomal evolution in culture in a negative fashion, possibly because of the widely held though largely unsubstantiated view that chromosome instability is the primary cause of the loss of morphogenetic potential in long term plant tissue cultures, and regeneration of plants may be useful for monitoring genetic changes of "*in vitro*" manipulations. It is significant that data opposing this view has arisen from a species with low chromosome number (Gould, 1978).

The induction of genic mutation in plant tissue cultures is a difficult area of study. A more positive approach to chromosomal mutation which normally occurs in culture may lead to the generation of novel karyotypes which could be useful for selection in somatic hybridization experiments.

CHAPTER 2

CELL CYCLE ANALYSIS OF THE CAPT SUSPENSION CELL CULTURE

2.1 INTRODUCTION

Cells in a long term culture system, are, for the main part, in a state of continual growth, which necessarily involves division. The division process is an ordered sequence of biochemical events, leading to mitosis, and separation of identical genomes into the 2 daughter cells produced. Mitosis is the only stage at which the chromatin is visually distinguishable in its functional subunits, the chromosomes. The alternative nuclear states, when chromatin is decondensed and dispersed, are collectively termed interphase. In 1952 Howard and Pelc discovered, by following the uptake of radioactivity labelled precursors that DNA was synthesised during a limited part of interphase and not during mitosis, as had been supposed. This led to the concept of the cell cycle, or, more specifically, the DNA replication-partition cycle during which the cell traverses a number of distinguishable phases between one mitosis and the next. The period of DNA synthesis is commonly designated the S phase. The interval between the previous mitosis and the S phase is called the G_1 phase, during which the cell may manifest its characteristic function, and the interval following S and leading into the next mitosis is called the G_2 phase. The symbols G_1 and G_2 merely stand for "gaps" in the proliferative cycle, although the use of such non-specific symbols should not conceal the fact that cellular events related specifically to the cycle must take place during this period.

The more recent ideas of Burns and Tannock (1970), and Smith and Martin (1973) divide the cell cycle into 2 phases, the A state, which

includes S, G_2, M and part of G_1 and the B state which is the part of G_1 which is variable in duration. This is referred to as the "transition probability" model, and was introduced to explain the observed variability in G_1 duration between cells in a given population. A cell's probability of leaving the A state, entering the B state, and hence proceeding through another division cycle is known as the transition probability. The G_1-S-G_2-M model and the transition probability model are not mutually exclusive. The more recent model merely places emphasis on the generally observed phenomenon that cells transit through the S-G_2-M sequence in a finite time period whereas progression through G_1 may be extended in duration, and some cells may never return to the cycling condition.

As noted in the General Introduction, these distinguishable cell cycle stages, which will all be represented in the rapidly growing cell suspension cultures used in this project, may confer characteristic properties on the cells. Such features may affect a cell's competence for transformation or fusion events. For example, possibly S phase cells, in which the DNA synthetic enzymes are activated, would more readily incorporate any introduced foreign DNA into the genome. Alternatively, differences in cell membrane properties through the cell cycle stages may affect the cell's potential for uptake of foreign genetic material. It is worth noting, at this point, that many previous DNA uptake studies with plants have utilised either whole plant material or leaf protoplasts, in which the cells will presumably be arrested at one stage of the cell cycle, the G_1 phase. Thus, cell cycle related phenomena would not be noticed in these systems.

Before analysing the affects of cell cycle stage on competence for

DNA uptake and fusion, it was considered important to establish the nature of the cell division cycle in the cultures to be used. Such analyses could give a description of a tissue culture population in terms of:

(1) the fraction of the population involved in the cell division process (the growth fraction),

(2) the fraction of cells in each cell cycle stage, and

(3) the duration of each cell cycle stage.

Such an analysis was completed on the HA-1 cell suspension culture by Gould (1977). This analysis demonstrated that greater than 95% of the culture population was involved in division, and the durations of each cycle phase at 26°C were as follows:- S, 3.5h; G_2, 2.5h; M, 2h; G_1, 20h. The G_1 phase was most variable in duration.

Cycle analysis has not been performed on a *Brachycome dichromosomatica* culture since the derived suspension cell cultures are very lumpy, and, also, a major fraction of cells is involved in xylogenesis. Both these characteristics make cell cycle analysis impractical, so that no attempt has been made to investigate features of cell proliferation in this species.

An analysis of the cell cycle in the tumour-derived CAPT suspension culture is presented here. Three methods of cell cycle analysis have been utilised, since no single analytical technique, applied in isolation, gives a satisfactory description of the dynamics of the cell cycle (Painter *et al.*, 1964; Mendelsohn and Takahashi, 1971; Gould *et al.*, 1974).

This analysis is of interest as an isolated piece of work, firstly because studies of cell division kinetics in plant tissue culture have

not progressed very far, with only a handful of reports published to date (for example, Ericksson, 1967; Gould *et al.*, 1974; Bayliss, 1975b; Chu and Lark, 1976; Gould, 1977). Secondly, this appears to be the first study of the cell cycle in plant tumours. Since a major feature of tumours is their capacity for essentially unrestrained or autonomous growth, it would seem that consideration of the kinetics of cell growth and division should form a major part of any description of the tumorous state. Certainly, characteristic changes in cycle duration and growth fraction occur in hyperplastic and neoplastic states in animals (Bresciani, 1968). Additionally, with one exception (Bayliss, 1975b) plant tissue cultures used in previous cell cycle studies, have all required the exogenous supply of at least one hormone, whereas the CAPT culture is auxin autotrophic.

2.2 MATERIALS AND METHODS

2.2.1 Estimation of Growth Parameters

Samples (1ml) were removed aseptically from CAPT cultures at the time of initiation of fresh cultures, and every 12 h. subsequently during the 7 day culture period. Both 10 ml and 20 ml innocula of cells were used for culture initiation each 7 days. The removed samples were then added to an equal volume of 10% chromium trioxide. Cells were macerated by heating this mixture at 60°C for 5 to 10 min. and cell lumps were dissociated by vigorous shaking for a similar period of time. Samples were then diluted and pipetted into the wells of specially designed counting slides. The field volume, viewed in the well with a Kyowa microscope at 100 X magnification, was calculated to be 0.26 µl. Dilutions

were performed to give about 50 cells per field volume. Estimations of the number of cells per ml of culture were made from counts of cells seen in 100 randomly selected fields. Means, and standard errors of the mean (S.E.M.) were calculated, and recounts were made where the S.E.M. was greater than 10%.

Growth curves were plotted of $\log_{10}$ cell number against hours of incubation, and population doubling times have been derived using mean counts of 4 (in the case of 20 ml innoculum) or 5 (in the case of 10 ml innoculum) separate cultures.

In addition, samples were scored for mitotic index and fraction of tracheid elements.

2.2.2 Labelling

Pulse labelling:-

CAPT cell suspension cultures were pulse labelled with tritiated thymidine (^{3}H Tdr) at 1 10^{-8} mol l^{-1} (specific activity 5 Ci m mol^{-1} [185 GBq m mol^{-1}] which gives an activity per ml of 5×10^{-2} µCi [1.85 kBq], supplied by Radiochemical Centre, Amersham) for 20 min, followed by a cold thymidine chase at 5×10^{-5} mol l^{-1}. Such a label-to-cold chase ratio of 1:5000 had previously been shown to be effective as a pulse labelling technique for both *Acer pseudoplatanus* suspension cell cultures (Gould *et al.*, 1974) and *Haplopappus gracilis* suspension cell cultures (Gould, 1977). The suitability of this labelling regime for CAPT cultures was tested by following the incorporation of a pulse of tritiated thymidine (1×10^{-8} mol l^{-1}, for 20 min) into the TCA (trichloracetic acid) insoluble fraction of CAPT cells for 4 h:

(i) in the absence of a cold thymidine chase, and

(ii) after a cold thymidine chase at three different levels (5×10^{-4} mol l^{-1}; 5×10^{-5} mol l^{-1}; 5×10^{-6} mol l^{-1}).

5 samples were removed from each of the 4 different treatments, over the 4 h period, and each mixed with an equal volume of 10% cold TCA, and left for 1 h to allow precipitation of macromolecules in the cells. Each sample was then filtered through a 0.45 μ millipore filter, and washed with 10 ml 5% TCA. The filters were then dried under a lamp, and added to 10 mls of scintillant (9.9 g PPO and 0.245 g POPOP in 1 L of toluene) in scintillation vials, ready for counting on a Beckman LS-250 Liquid Scintillation System. Duplicate counts, each for a 5 min period, were made on each sample, and mean counts per min per ml were calculated, and plotted against time after initial pulse labelling treatment.

Flash labelling:-

CAPT cell suspensions were treated with ^{3}H Tdr at 1×10^{-8} mol l^{-1}, as above, for 20 min, then fixed in 3:1 ethanol:acetic acid ready for cytological preparation.

Continuous labelling:-

CAPT cell suspensions were repeatedly supplied with ^{3}H Tdr (1×10^{-8} mol l^{-1}, every 6 h). Preliminary investigations were performed, to ensure that, under this labelling regime, cells would incorporate titium into their macromolecular fraction, in an essentially linear fashion, throughout the experimental period. Cell cultures were used during the exponential growth period, and sampling was continued for 48 h. It was necessary to sample over such a long period for analysis of cell

cycle parameters, this time being slightly longer than the apparent doubling time (see Results Section) for CAPT cultures. Using the TCA precipitation techniques described under pulse-labelling methodology, incorporation of ^{3}H Tdr, at two different levels (1×10^{-8} mol l^{-1}; 1×10^{-6} mol l^{-1}) was investigated. In a second experiment, cultures were pulsed with ^{3}H Tdr at 1×10^{-8} mol l^{-1} every 6 h or 12 h. Additionally, in the second experiment, the total cellular label incorporation, and the label remaining in the culture medium, were measured at each sample time. For estimation of total cellular label, samples were filtered and washed with 10 ml fresh medium (B5-2,4-D) containing cold thymidine (at 1000 X original ^{3}H Tdr). Filters were then dried, and placed in 10 ml scintillant ready for counting, as for the TCA insoluble fraction. For estimation of the amount of label remaining in the medium, 0.2 ml of culture supernatants was added directly to 10 ml scintillant for counting. Such multiple analysis of labelling patterns in the cells yields information on the dynamic features of titium incorporation, allowing estimates of cellular thymidine pool size.

Estimation of the degree of quenching:-

Quench is defined as any process that reduces the photon output of the scintillation liquid, and, hence, the count rate reported for the sample under investigation. It was thus necessary to measure the degree of quenching occurring in samples prepared for the determination of TCA insoluble, or total cellular activity in cell samples. Standard vials, containing known levels of ^{3}H Tdr (from 0.05µCi to 0.25µCi) in 10 ml scintillant, were counted. Filters were then prepared, using unlabelled CAPT cells, and following the procedures for TCA insoluble or total cellular determination. These filters were then added to the standard

vials, and recounts were made. Counts were made in duplicate, and means were plotted against ^{3}H Tdr level.

2.2.3 S-Phase Analysis

Successful analysis of the cell cycle by the following methods relies on all S-phase cells in a population incorporating similar amounts of ^{3}H Tdr during flash labelling treatments. In other words, DNA synthesis should be continuous, and should occur at a fairly steady rate throughout the S-phase period. Analysis of S-phase was therefore performed on CAPT cultures. Using autoradiographs of flash labelled cell populations, densitometry techniques were used to correlate DNA contents of S-phase cells with silver grain density overlying the nuclei. If the rate of DNA synthesis varies greatly during S-phase, this would be detected as increased grain density over nuclei of certain DNA contents. Alternatively, if DNA synthesis is discontinuous, and stops at certain stages during S-phase, no, or very little grain will occur over nuclei of particular DNA contents.

Techniques for automatic, quantitative estimation of grain density in autoradiographs, described in detail in the General Materials and Methods, were employed in this investigation. Comparisons were made using both Farmer's Reducer method and the Two Wavelength method.

2.2.4 Mitotic and Labelling Indices

Mitotic and labelling indices, determined on populations of flash labelled cells, were means of counts of close to 3000 nuclei.

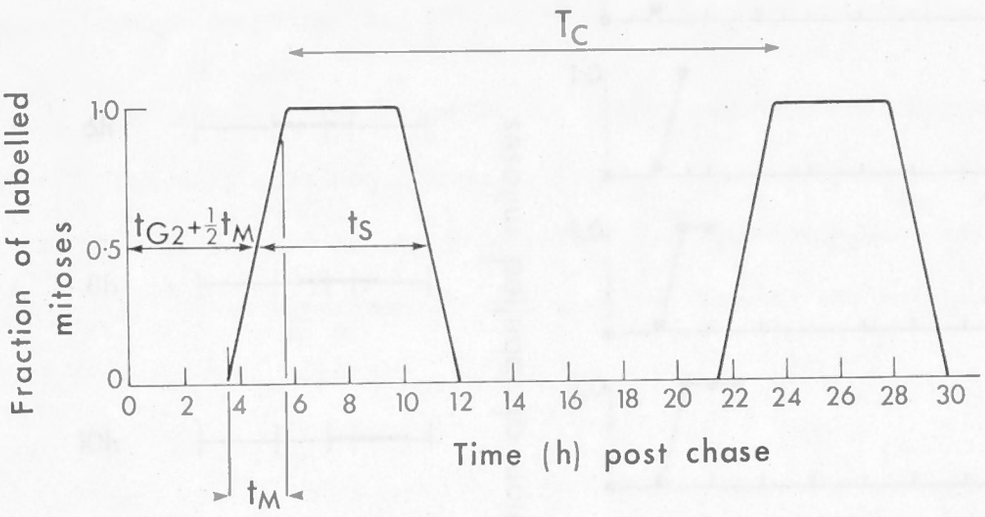
2.2.5 Cell Cycle Analyses

Three methods of cell cycle analysis were performed on CAPT suspension cell cultures.

Fraction of labelled mitoses method:-

For FLM analysis on CAPT cultures cell suspensions were labelled by the pulse-chase method previously described, and samples were removed every 1, or 1.5 h, and fixed in 3:1 ethanol:acetic acid ready for preparation of Feulgen stained autoradiographs. By this method, only those cells which are in the DNA synthetic period (S-phase) will incorporate label into their nuclei during the 20 min pulse period. This labelled population of cells is then followed, as it progresses through the cell cycle, and enters mitosis, by measuring the fraction of labelled mitoses with the passage of time. Mitosis, unlike other phases of the cell cycle, is cytologically distinguishable, and therefore provides a window through which the progress of the labelled cohort can be observed. In an idealised situation where no variations in cycle phase durations occur waves of labelled mitoses occur, as illustrated in Fig. 2.1, and cycle phase durations can be estimated, as shown. Fig. 2.2 demonstrates how such a curve is generated. In real populations, each phase varies around a mean value, producing damping of the FLM curve. Thus, durations of the cell cycle, and its phases are usually read from 0.5 levels, giving values approximating to the median values for the population under study.

FLM parameters for CAPT populations, were calculated from observations of 100 mitotic figures for each data point. Phase durations were determined both manually, as illustrated in Fig. 2.1, and also by computer analysis,



t_M = mitotic duration

t_S = S phase duration

t_{G2} = G_2 duration

T_C = total cycle time

t_{G1} (G_1 duration) = $T_C - (t_M + T_S + t_{G2})$

FIG. 2.1 Idealised FLM curve, showing the derivation of the individual cell cycle parameters directly from the curve.

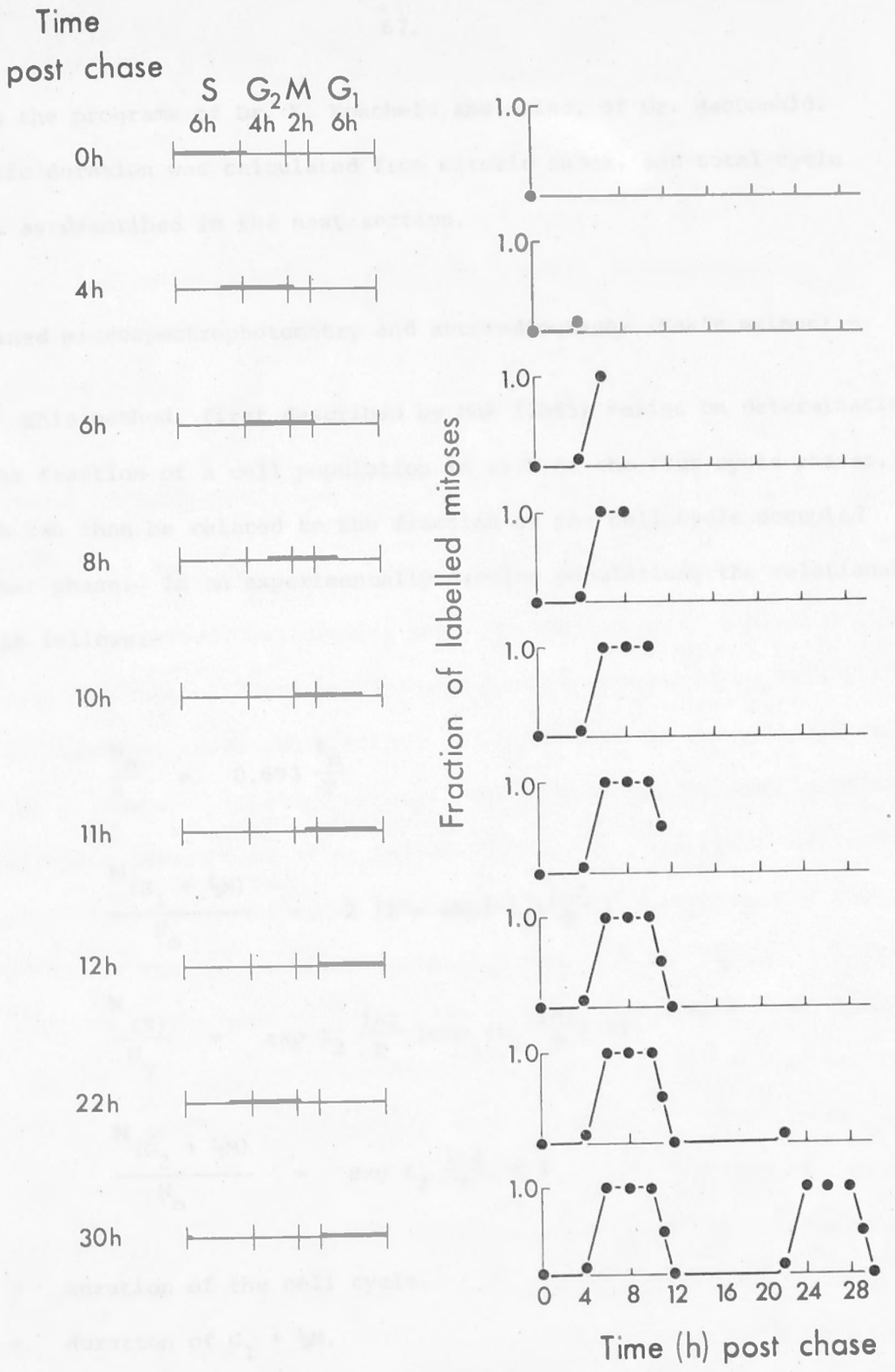


FIG. 2.2 A diagrammatic representation of the generation of an FLM curve, modelled on a cycle time of 18 h, with S = 6 h; G_2 = 4 h; M = 2 h and G_1 = 6 h. The position in the cell cycle of the labelled cohort of cells (pulse-chase labelling being performed at 0 h) at various times post chase is indicated in the left hand diagram by a thick line. On the right hand side of the diagram the corresponding FLM curve for each time is shown.

using the programs of Dr. K. Koschel, and, also, of Dr. MacDonald. Mitotic duration was calculated from mitotic index, and total cycle time, as described in the next section.

Combined microspectrophotometry and autoradiography (Mak's method):-

This method, first described by Mak (1965) relies on determination of the fraction of a cell population in each of the four cycle phases, which can then be related to the fraction of the cell cycle occupied by that phase. In an experimentally growing population, the relationships are as follows:-

$$\frac{N_m}{N_o} = 0.693 \frac{t_m}{T}$$

$$\frac{N_{(G_1 + \frac{1}{2}M)}}{N_o} = 2 \left[1 - \exp(-t_1)\frac{\ln 2}{T}\right]$$

$$\frac{N_{(S)}}{N_o} = \exp t_2 \frac{\ln 2}{T} \left[\exp \left(t_s \frac{\ln 2}{T}\right) - 1\right]$$

$$\frac{N_{(G_2 + \frac{1}{2}M)}}{N_o} = \exp t_2 \frac{\ln 2}{T} - 1$$

T = duration of the cell cycle.

t_1 = duration of $G_1 + \frac{1}{2}M$.

t_2 = duration of $G_2 + \frac{1}{2}M$.

t_s = duration of S-phase.

N_o = number of cells in population.

t_m = duration of mitosis

In the CAPT suspension cultures, the ratio of G_1 to G_2 nuclei was estimated from DNA distributions of 200 unlabelled interphase nuclei, after flash labelling. Using this ratio, the relevant mitotic and labelling indices, and the total cycle time, as determined by another method, the durations of each cell cycle phase were determined, using the above formulae.

Continuous labelling method:-

CAPT cell suspensions were pulsed with ^{3}H Tdr every 6 h, as previously described, and samples were removed over a 45 h period, and fixed in 3:1 ethanol:acetic acid ready for preparation of Feulgen stained autoradiographs. Both the fraction of labelled cells (labelling index), and the fraction of labelled mitoses, were determined for each sample, and plotted against time after initial labelling. Labelling indices were determined on counts of 2,000 nuclei, and FLM parameters on 100 mitotic cells per data point. Cells in S-phase at the time of initial labelling will incorporate ^{3}H Tdr, and, since this label is continuously supplied, all cycling cells eventually enter S-phase and become labelled, so that a plateau level of labelled cells is reached. If all cells in the population are in cycle (*i.e.* growth fraction = 1), then the entire population will become labelled in a time equal to the mean durations of $t_{G_2} + t_M + t_{G_1}$ (see Fig. 2.3). Continuous labelling may be used to determine the growth fraction. From the plot of labelled mitoses, the time taken to reach 100% mitotic cells labelled is equivalent to $t_{G_2} + t_M$.

An additional analysis was performed on a culture which was pulsed just once with ^{3}H Tdr.

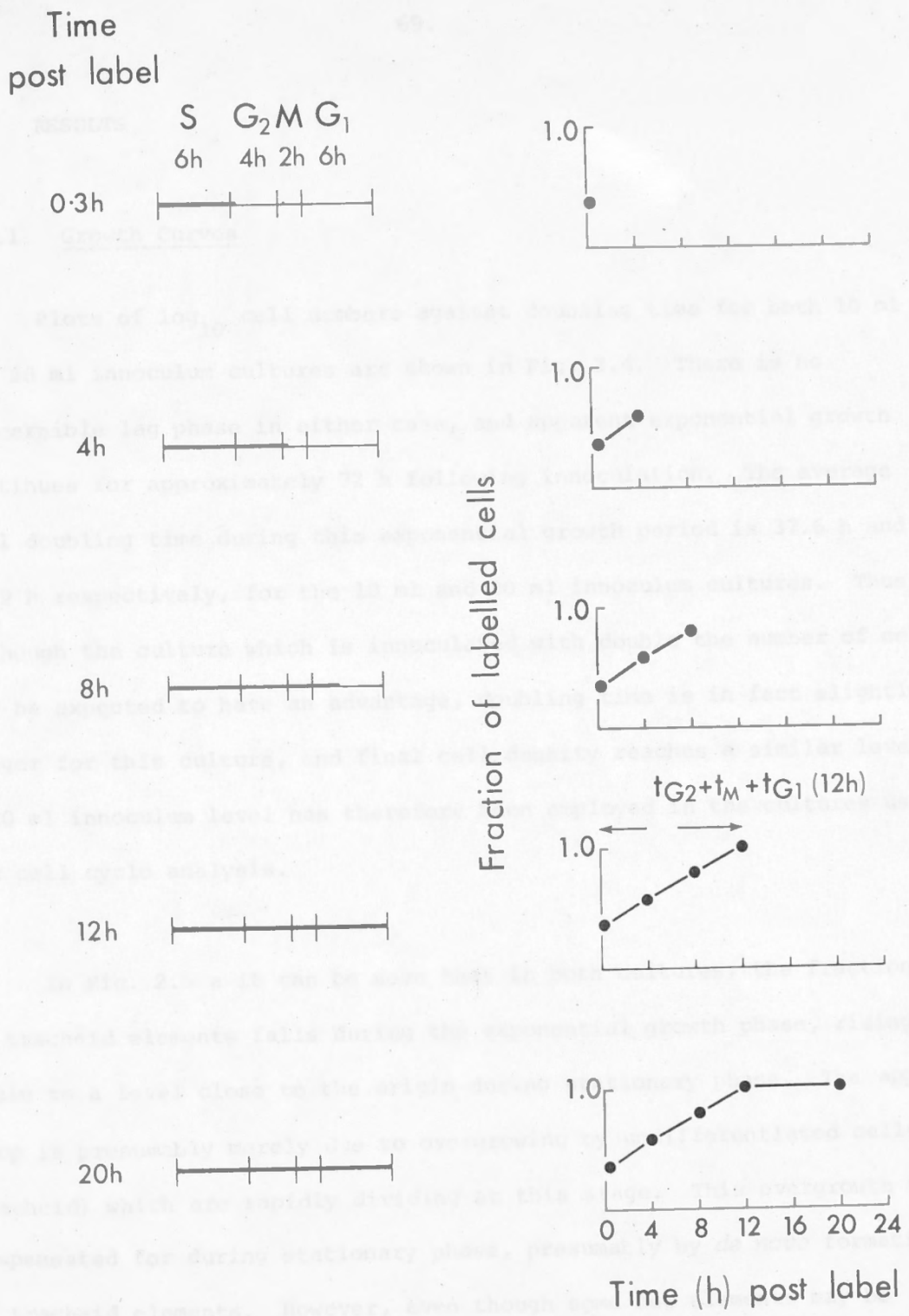


FIG. 2.3 A diagrammatic representation of the generation of a "continuous labelling" curve modelled on a cycle time of 18 h, with S = 6 h; G_2 = 4 h; M = 2 h; and G_1 = 6 h. On the left hand side of the diagram the fraction of cells labelled, and their position in the cell cycle, at various times post initial labelling is indicated by a thick line. On the right hand side of the diagram the plot of fraction of labelled cells at these various times is shown. The time taken for all cells to become labelled is equal to $t_{G_2} + t_M + t_{G_1}$ (*i.e.* $T_c - t_s$) as shown.

2.3 RESULTS

2.3.1 Growth Curves

Plots of $\log_{10}$ cell numbers against doubling time for both 10 ml and 20 ml innoculum cultures are shown in Fig. 2.4. There is no discernible lag phase in either case, and apparent exponential growth continues for approximately 72 h following innoculation. The average cell doubling time during this exponential growth period is 37.6 h and 42.9 h respectively, for the 10 ml and 20 ml innoculum cultures. Thus, although the culture which is innoculated with double the number of cells may be expected to have an advantage, doubling time is in fact slightly longer for this culture, and final cell density reaches a similar level. A 10 ml innoculum level has therefore been employed in the cultures used for cell cycle analysis.

In Fig. 2.5 a it can be seen that in both cultures, the fraction of tracheid elements falls during the exponential growth phase, rising again to a level close to the origin during stationary phase. The apparent drop is presumably merely due to overgrowing by undifferentiated cells (non-tracheid) which are rapidly dividing at this stage. This overgrowth is later compensated for during stationary phase, presumably by *de novo* formation of tracheid elements. However, even though some new elements may be formed during stationary phase, the fraction in the culture line obviously remains low and fairly constant from each 7 day regime to the next. This level of low leakage of cells into a pathway of cytodifferentiation will have little effect on the cell cycle analyses.

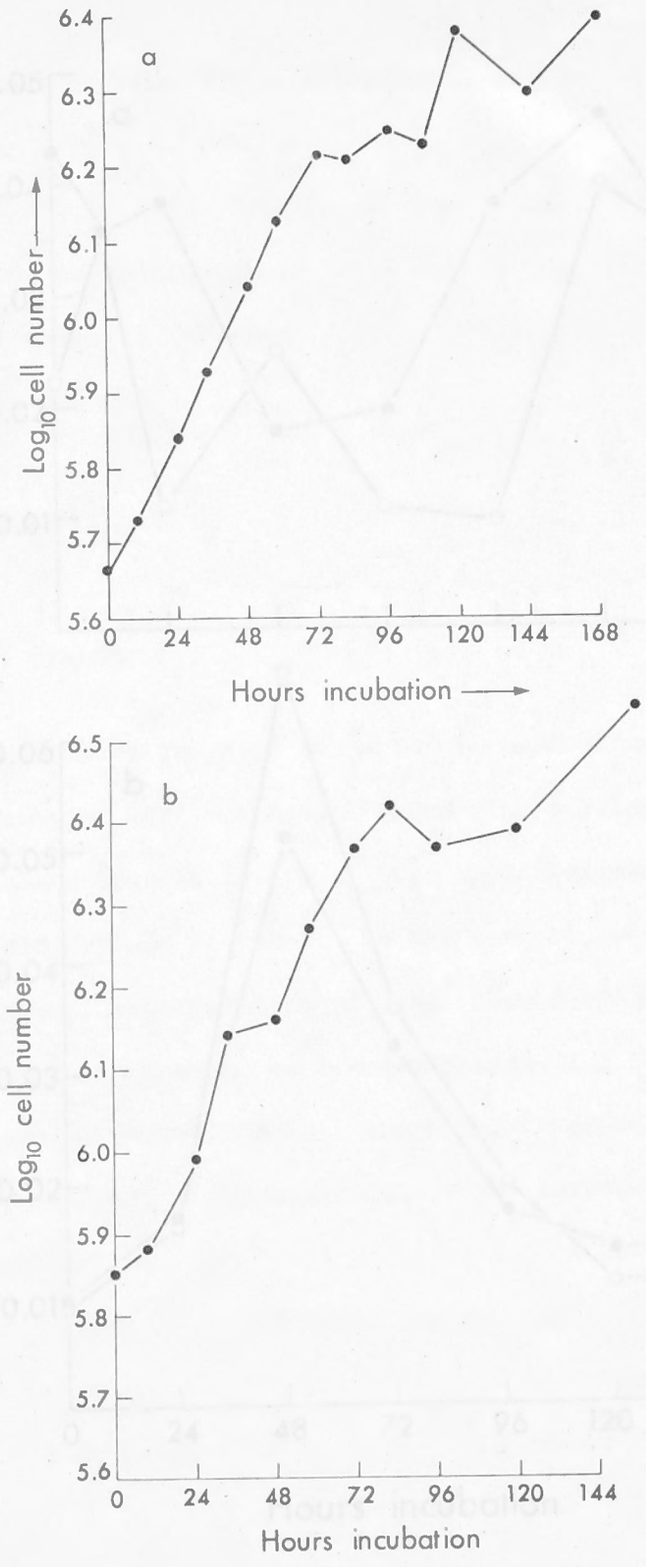


FIG. 2.4 Plots of $\log_{10}$ cell number against time of incubation of suspension cell cultures of CAPT. Plot a. is derived from means of counts on 5 different cultures, having an initial innoculum volume of 10 ml 7 days old culture into 50 ml fresh medium. Plot b. is derived from means of counts on 4 different cultures having an initial innoculum volume of 20 ml 7 day old culture into 50 ml fresh medium.

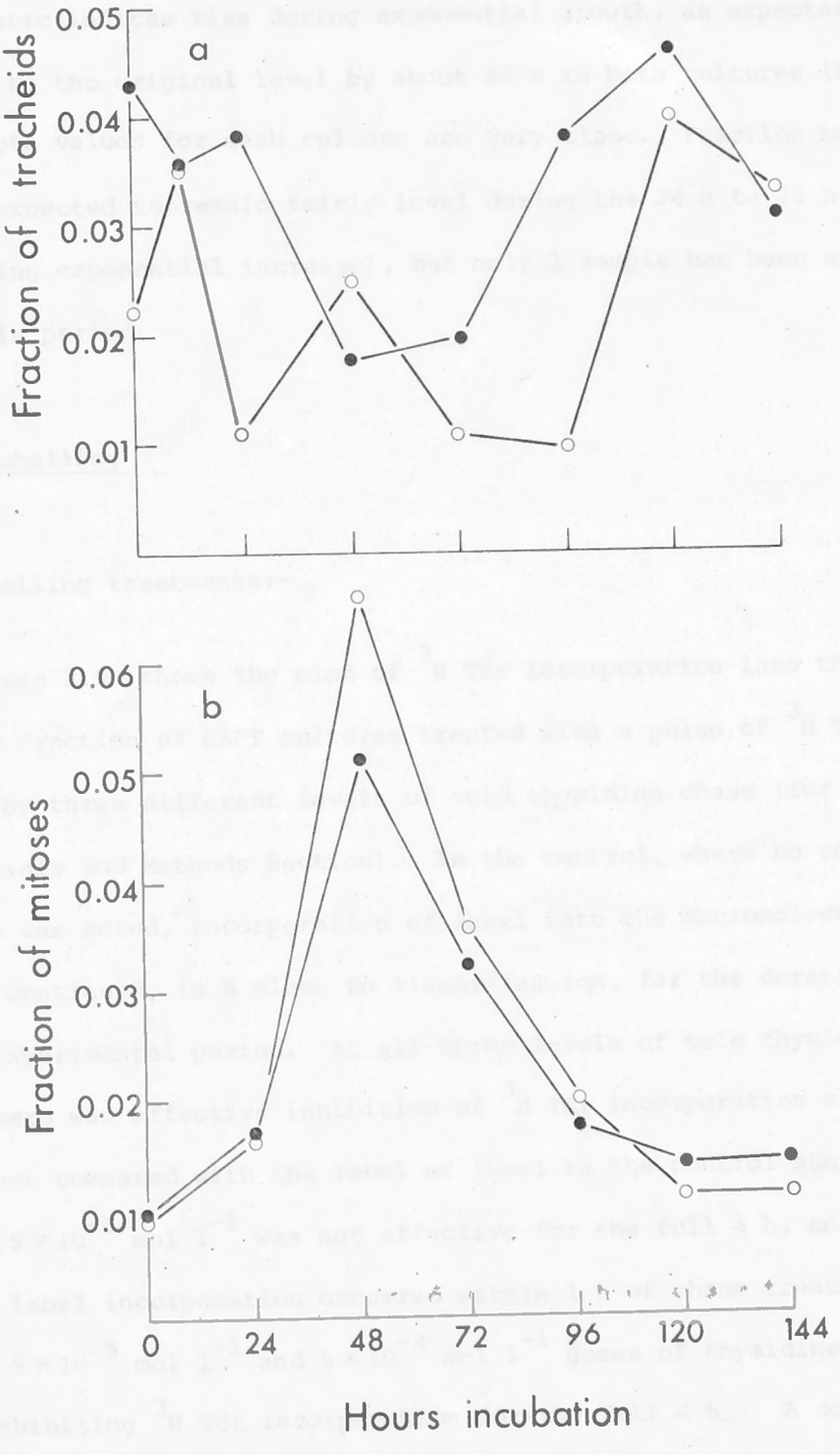


FIG. 2.5 Plots of (a) fraction of tracheids, and (b) fraction of mitoses against time of incubation in 2 (10 ml innoculum) CAPT suspension cell cultures. The individual plots in each figure represent the 2 separate cultures.

Mitotic indices rise during exponential growth, as expected, returning to the original level by about 96 h in both cultures (Fig. 2.5b). The absolute values for each culture are very close. Fraction of mitoses would be expected to remain fairly level during the 24 h to 72 h period (i.e. during exponential increase), but only 1 sample has been analysed during this period.

2.3.2 Labelling

Pulse labelling treatments:-

Figure 2.6 shows the plot of ^{3}H Tdr incorporation into the TCA insoluble fraction of CAPT cultures treated with a pulse of ^{3}H Tdr followed by three different levels of cold thymidine chase (for details see Materials and Methods Section). In the control, where no cold thymidine was added, incorporation of label into the macromolecular fraction continued, in a close to linear fashion, for the duration of the 4 h experimental period. At all three levels of cold thymidine chase, there was effective inhibition of ^{3}H Tdr incorporation after only 5 min, when compared with the level of label in the control sample. The chase of 5×10^{-6} mol l^{-1} was not effective for the full 4 h, and low rates of label incorporation occurred within 1 h of chase treatment. Both the 5×10^{-5} mol l^{-1} and 5×10^{-4} mol l^{-1} doses of thymidine were effective in inhibiting ^{3}H Tdr incorporation for the full 4 h. A cold chase of 5×10^{-5} mol l^{-1} thymidine was, therefore used in FLM analyses, as detailed in the Materials and Methods Section.

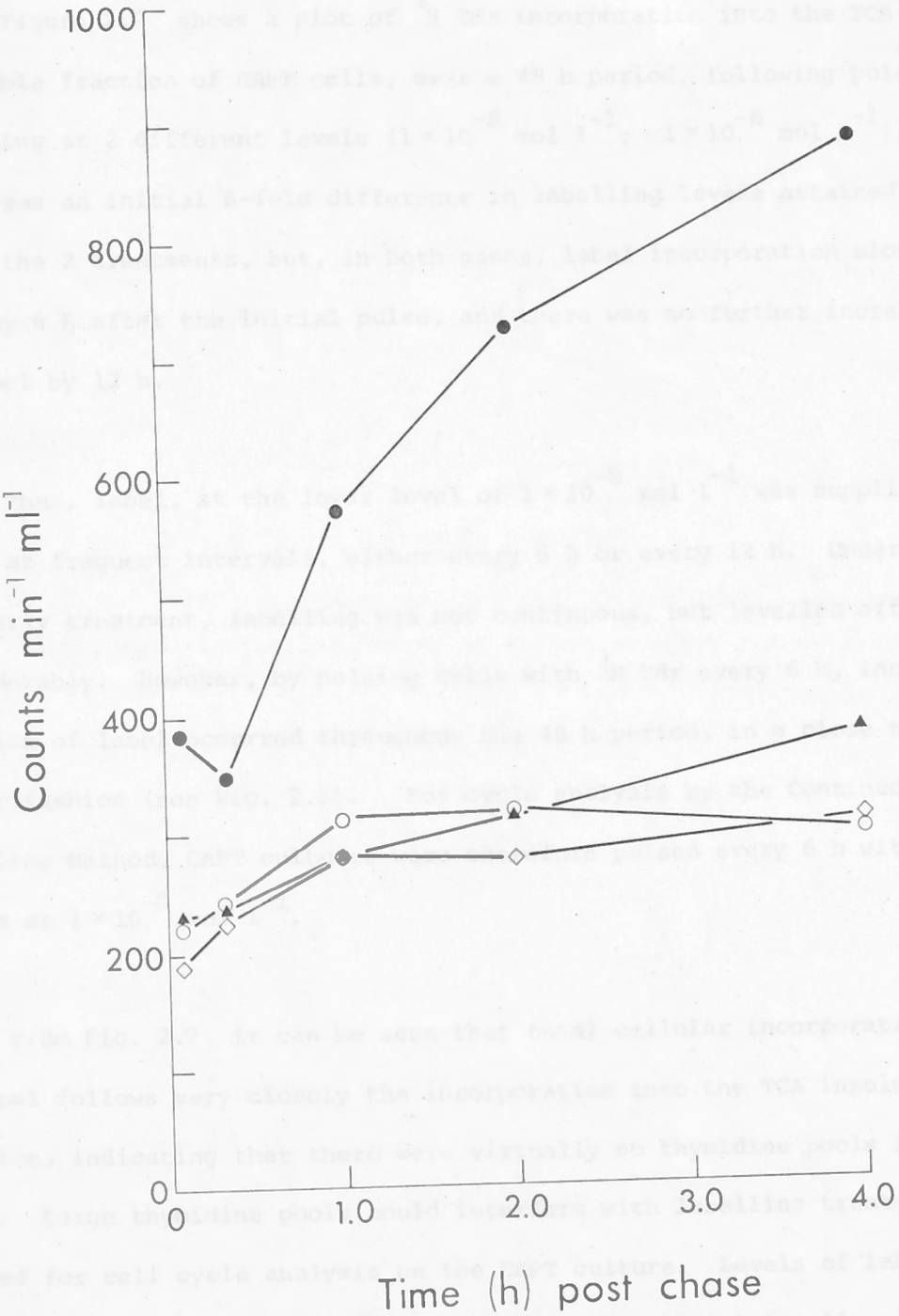


FIG. 2.6 A plot of counts in the insoluble fraction of a CAPT suspension cell culture following labelling with ^{3}HTdr and chasing with cold thymidine at 3 different levels. —●— Control (*i.e.* no chase); —▲— chase at 5×10^{-6} M; —◇— 5×10^{-5}M; —o— 5×10^{-4}M.

Continuous labelling treatments:-

Figure 2.7 shows a plot of ^{3}H Tdr incorporation into the TCA insoluble fraction of CAPT cells, over a 48 h period, following pulse labelling at 2 different levels (1×10^{-8} mol l^{-1}; 1×10^{-6} mol l^{-1}). There was an initial 6-fold difference in labelling levels attained under the 2 treatments, but, in both cases, label incorporation slowed down by 6 h after the initial pulse, and there was no further increase in label by 12 h.

Thus, label, at the lower level of 1×10^{-8} mol l^{-1} was supplied to cells at frequent intervals, either every 6 h or every 12 h. Under the 12 hourly treatment, labelling was not continuous, but levelled off considerably. However, by pulsing cells with ^{3}H Tdr every 6 h, incorporation of label occurred throughout the 48 h period, in a close to linear fashion (see Fig. 2.8). For cycle analysis by the Continuous Labelling Method, CAPT cultures were therefore pulsed every 6 h with ^{3}H Tdr at 1×10^{-8} mol l^{-1}.

From Fig. 2.9 it can be seen that total cellular incorporation of label follows very closely the incorporation into the TCA insoluble fraction, indicating that there were virtually no thymidine pools in the cells. Large thymidine pools could interfere with labelling treatments as used for cell cycle analysis on the CAPT culture. Levels of label in the medium followed a step-wise pattern, increasing initially with every pulse, then being depleted, to some extent, before the next pulse. However, the level of label in the medium always remained higher than the level in either the TCA insoluble or total cellular fraction.

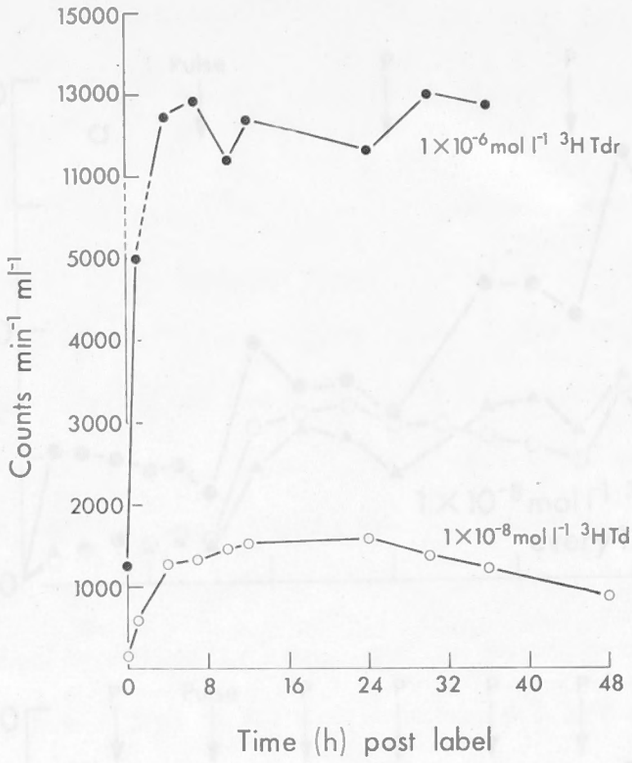


FIG. 2.7 ^{3}HTdr incorporation into the TCA insoluble fraction of CAPT suspension cell cultures against time after pulse labelling at 2 different levels (1×10^{-6} mol l^{-1}; 1×10^{-8} mol l^{-1}).

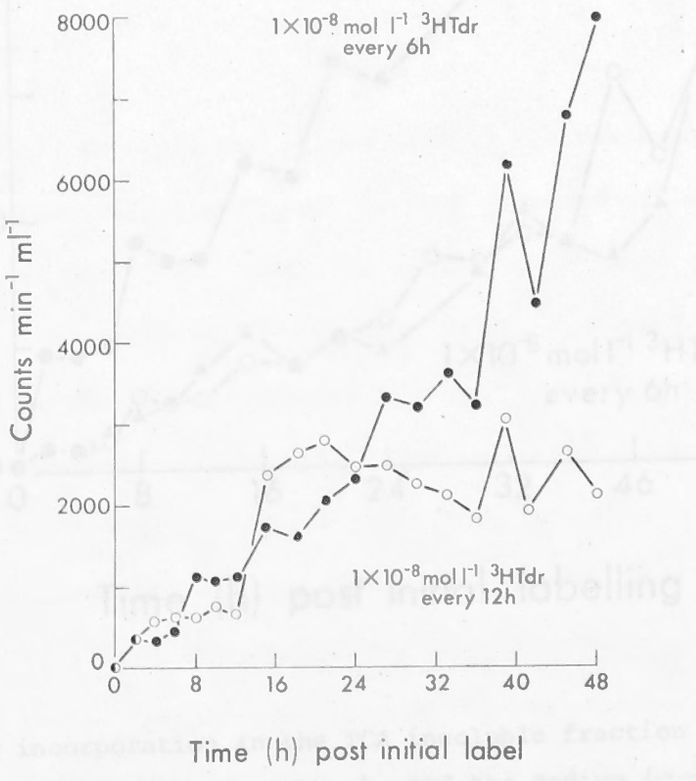


FIG. 2.8 ^{3}HTdr incorporation into the TCA insoluble fraction of CAPT suspension cell cultures against time. ——●—— labelling at 1×10^{-8} mol l^{-1} every 6 h. ——o—— labelling at 1×10^{-8} mol l^{-1} every 12 h.

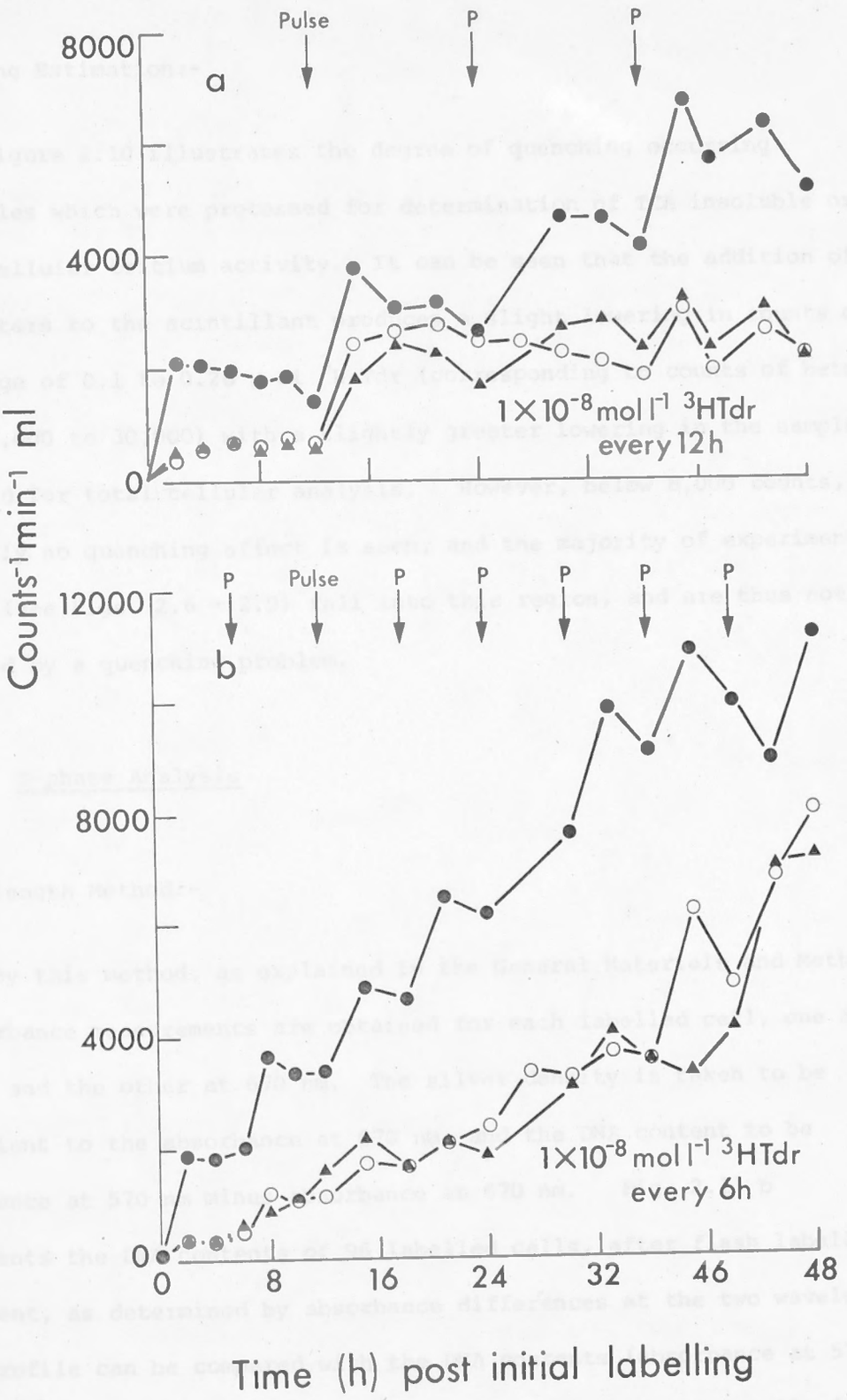


FIG. 2.9 ^{3}HTdr incorporation in the TCA insoluble fraction (——o——), the total cellular fraction (——▲——), and the medium (——●——) of 2 CAPT cell suspension cultures pulse labelled with (a) 1×10^{-8} mol l^{-1} ^{3}HTdr every 12 h and (b) 1×10^{-8} mol l^{-1} ^{3}HTdr every 6 h.

Quenching Estimation:-

Figure 2.10 illustrates the degree of quenching occurring in samples which were processed for determination of TCA insoluble or total cellular tritium activity. It can be seen that the addition of the filters to the scintillant produces a slight lowering in counts over the range of 0.1 to 0.28 μ Ci ^{3}H Tdr (corresponding to counts of between about 7,000 to 30,000) with a slightly greater lowering in the samples prepared for total cellular analysis. However, below 8,000 counts, virtually no quenching effect is seen, and the majority of experimental values (see Figs. 2.6 → 2.9) fall into this region, and are thus not affected by a quenching problem.

2.3.3 S-phase Analysis

2-Wavelength Method:-

By this method, as explained in the General Materials and Methods, 2 absorbance measurements are obtained for each labelled cell, one at 570 nm and the other at 670 nm. The silver density is taken to be equivalent to the absorbance at 670 nm, and the DNA content to be absorbance at 570 nm minus absorbance at 670 nm. Fig. 2.11 b represents the DNA contents of 96 labelled cells, after flash labelling treatment, as determined by absorbance differences at the two wavelengths. This profile can be compared with the DNA contents (absorbance at 570 nm) of 96 unlabelled cells from the same slide. The unlabelled cells fall into two distinct peaks, one ranging from 600 to 1400 (taken to be the G_1 cells), and the other ranging from 1600 to 2800 (taken to be the G_2 cells), leaving a gap where the S-phase cells presumably fall. Certainly,

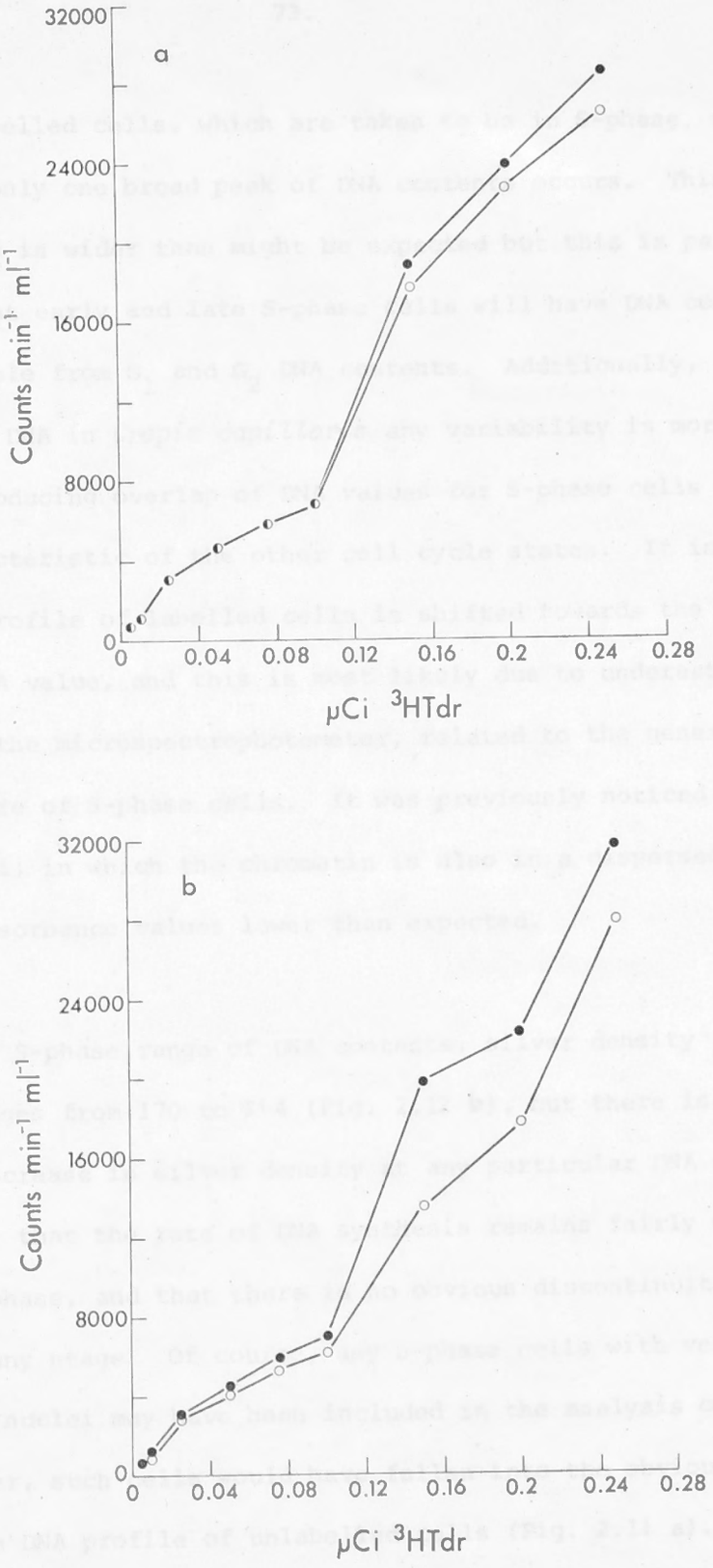


FIG. 2.10 Degree of quenching by (a) TCA insoluble method, ——●—— label + scintillant, ——o—— label + scintillant + filter; (b) total cellular activity method, ——●—— label + scintillant, ——o—— label + scintillant + filter.

many of the labelled cells, which are taken to be in S-phase, fall into this gap, and only one broad peak of DNA contents occurs. This range of DNA contents is wider than might be expected but this is partly due to the fact that early and late S-phase cells will have DNA contents indistinguishable from G_1 and G_2 DNA contents. Additionally, due to the low content of DNA in *Crepis capillaris* any variability is more noticeable, producing overlap of DNA values for S-phase cells with DNA contents characteristic of the other cell cycle states. It is noticed that the DNA profile of labelled cells is shifted towards the G_1 rather than the G_2 DNA value, and this is most likely due to underestimation of absorbance by the microspectrophotometer, related to the generally dispersed nature of S-phase cells. It was previously noticed that prophase nuclei, in which the chromatin is also in a dispersed state often yield absorbance values lower than expected.

Over the S-phase range of DNA contents, silver density (absorbance at 670 nm) ranges from 170 to 814 (Fig. 2.12 b), but there is no marked increase or decrease in silver density at any particular DNA content. This indicates that the rate of DNA synthesis remains fairly steady throughout S-phase, and that there is no obvious discontinuity of DNA synthesis at any stage. Of course, any S-phase cells with very little label overlying the nuclei may have been included in the analysis of unlabelled cells. However, such cells would have fallen into the obvious gap which appears in the DNA profile of unlabelled cells (Fig. 2.11 a).

There is good correlation between visual grain counts and the silver density as quantified by the 2 wavelength method (Fig. 2.13a).

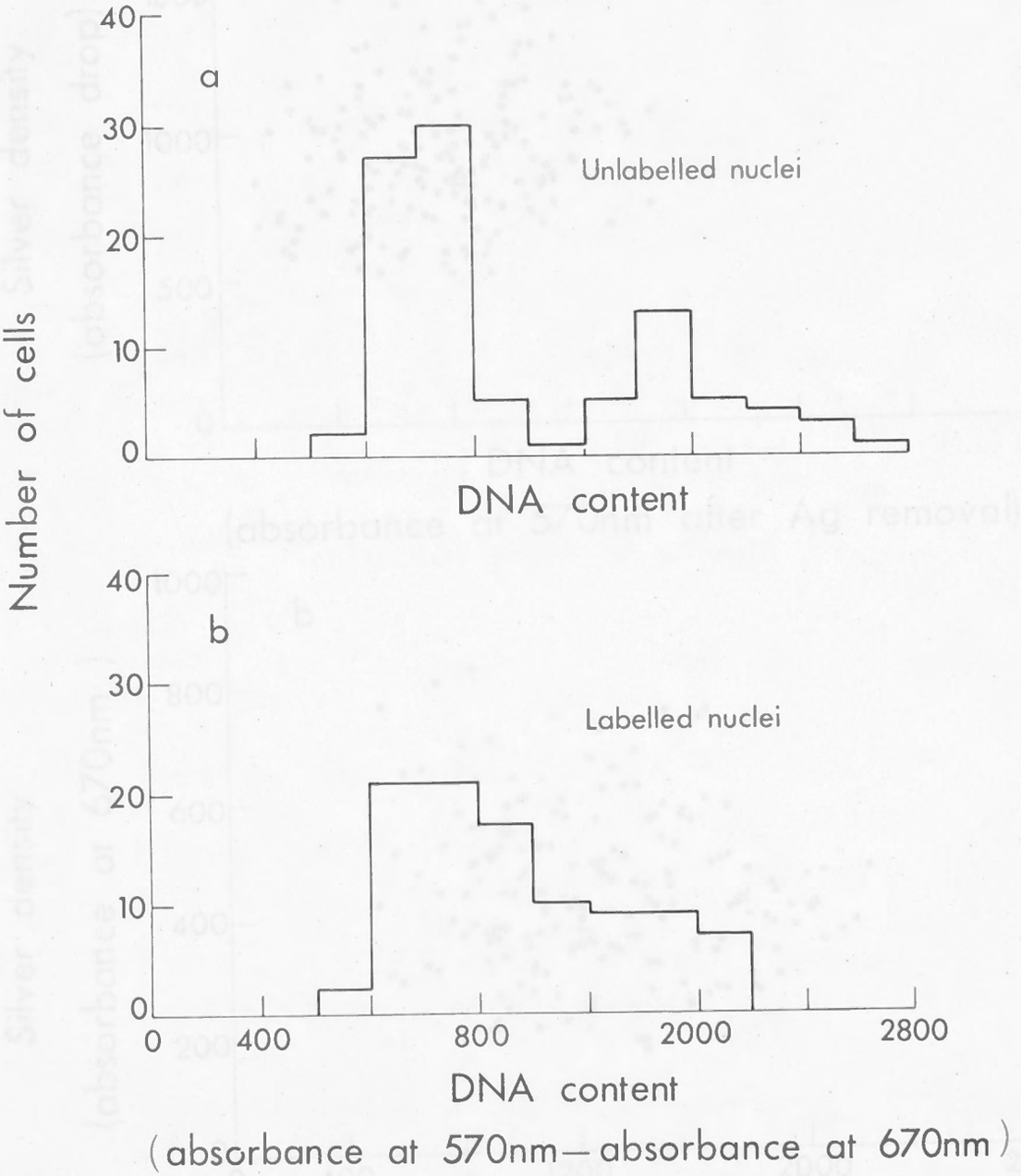


FIG. 2.11 DNA contents of a population of pulse-chase labelled CAPT cells. (a) 96 unlabelled cells; (b) 96 labelled cells, measured by using the 2-wavelength method.

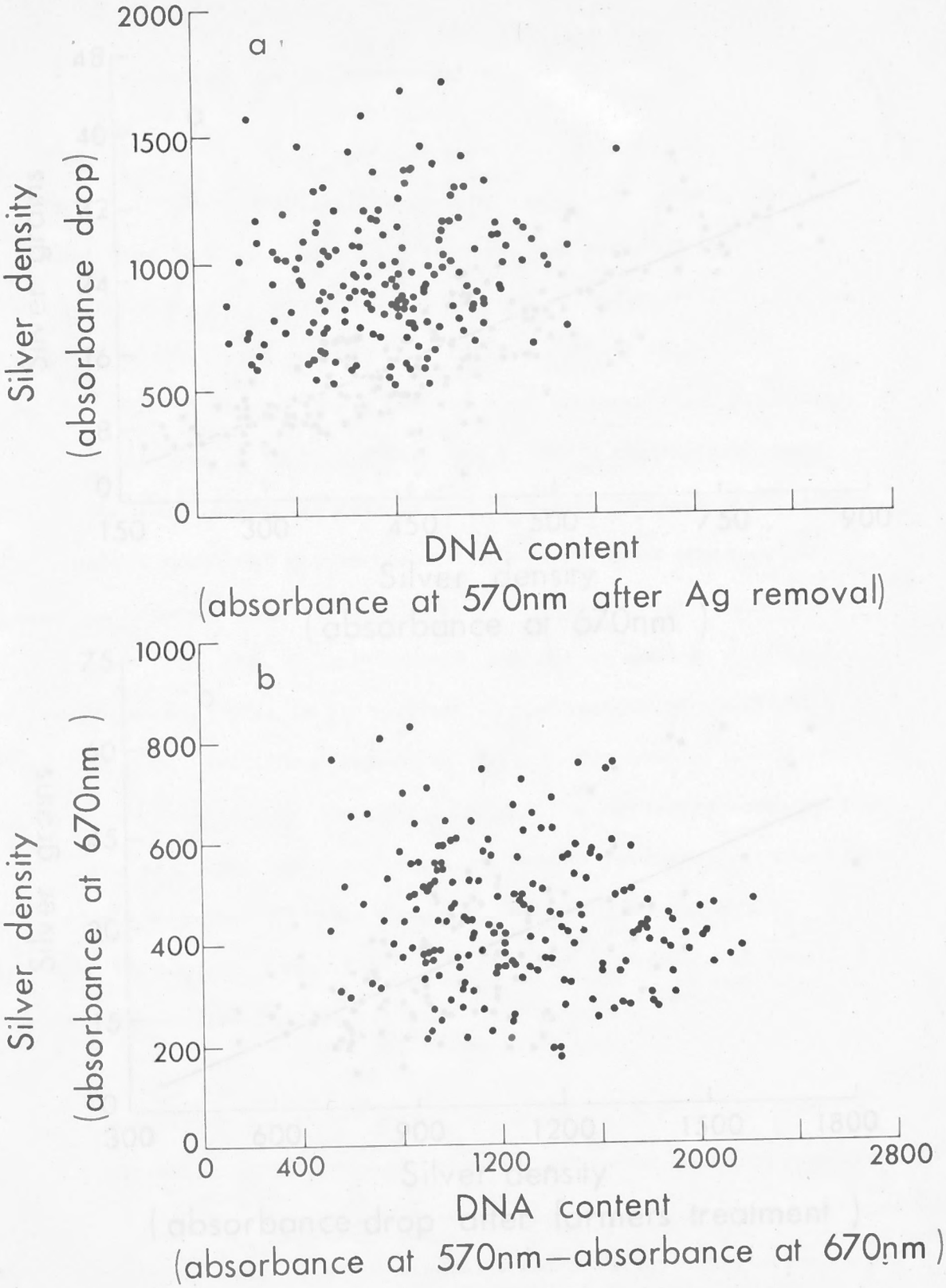


FIG. 2.12 Silver density for individual cells within the S-phase range of DNA contents as measured by (a) silver removal method, and (b) 2-wavelength method.

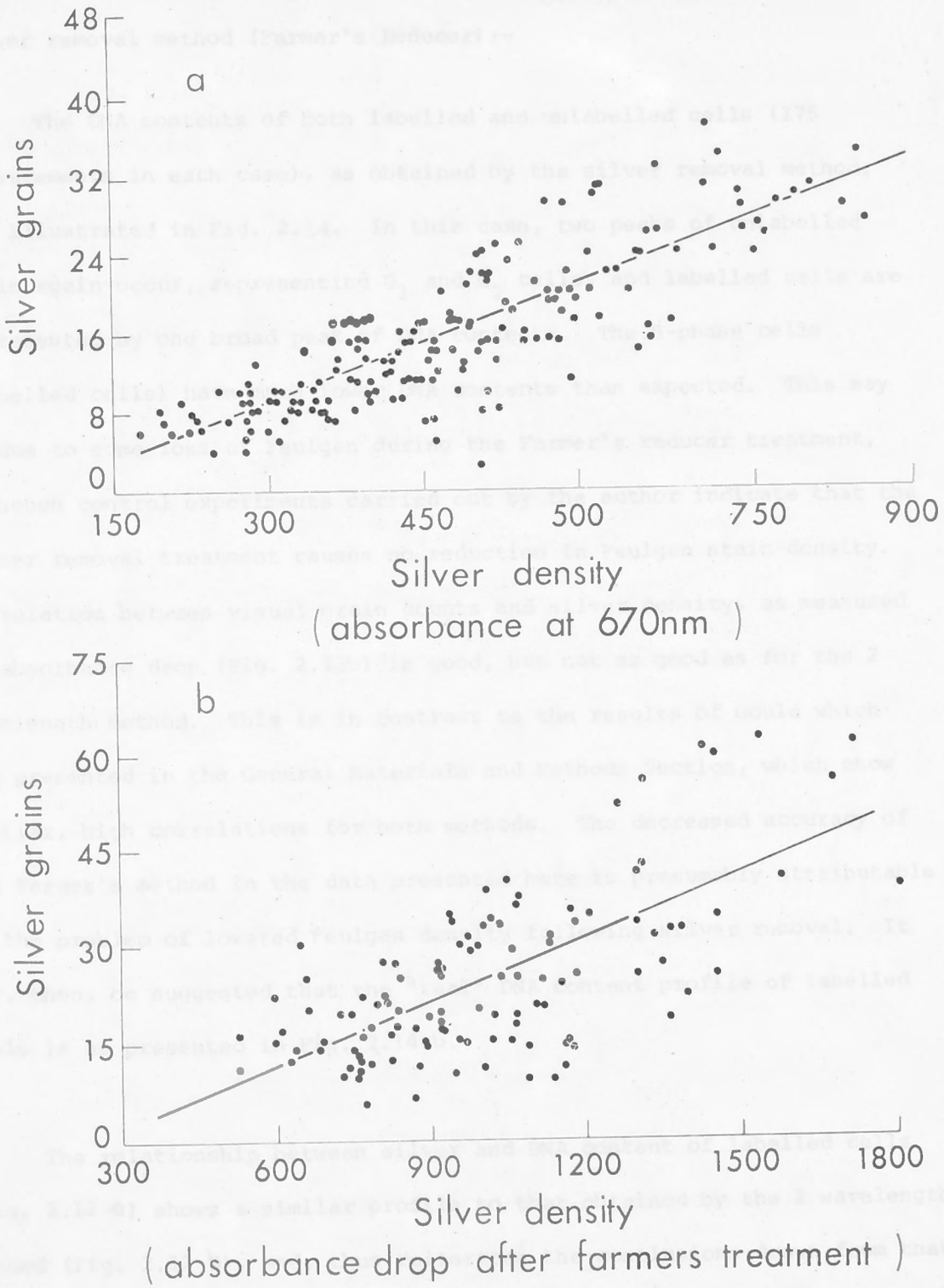


FIG. 2.13 Correlations between silver grain counts and silver densities obtained by quantitative microspectrophotometry by (a) 2-wavelength method, and (b) silver removal method.

Silver removal method (Farmer's Reducer):-

The DNA contents of both labelled and unlabelled cells (175 measurements in each case), as obtained by the silver removal method, are illustrated in Fig. 2.14. In this case, two peaks of unlabelled cells again occur, representing G_1 and G_2 cells, and labelled cells are represented by one broad peak of DNA contents. The S-phase cells (labelled cells) have much lower DNA contents than expected. This may be due to some loss of Feulgen during the Farmer's reducer treatment, although control experiments carried out by the author indicate that the silver removal treatment causes no reduction in Feulgen stain density. Correlation between visual grain counts and silver density, as measured by absorbance drop (Fig. 2.13b) is good, but not as good as for the 2 wavelength method. This is in contrast to the results of Gould which are presented in the General Materials and Methods Section, which show similar, high correlations for both methods. The decreased accuracy of the Farmer's method in the data presented here is presumably attributable to the problem of lowered Feulgen density following silver removal. It may, then, be suggested that the "real" DNA content profile of labelled cells is as presented in Fig. 2.14 b.

The relationship between silver and DNA content of labelled cells (Fig. 2.12 **a**) shows a similar profile to that obtained by the 2 wavelength method (Fig. 2.12 **b**), and, thus reiterates the conclusions drawn from that data *i.e.* that the rate of DNA synthesis remains fairly constant, with no obvious non-synthetic periods, throughout the S-phase.

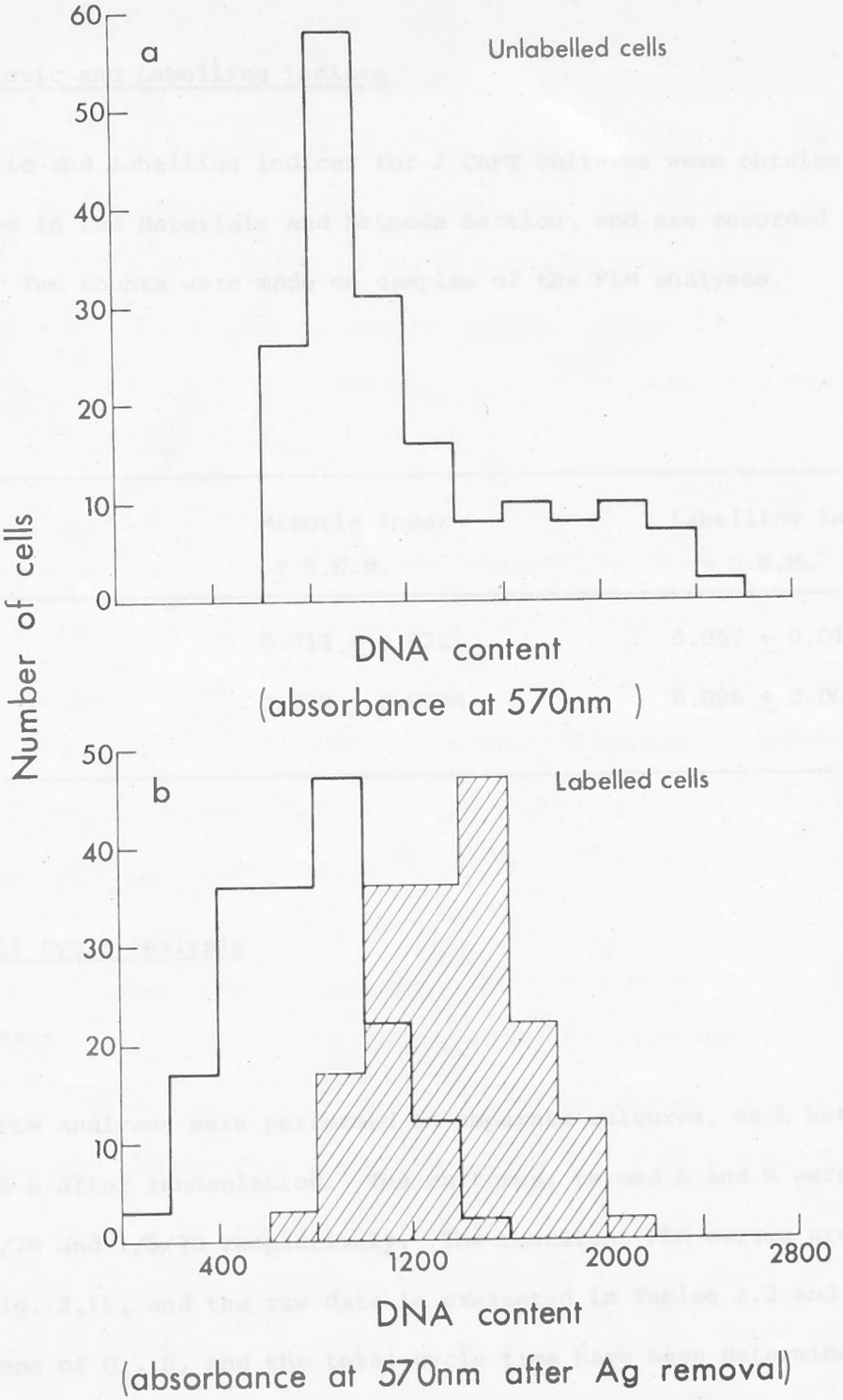


FIG. 2.14 DNA contents of a population of pulse-chase labelled CAPT cells, (a) 175 unlabelled cells, (b) 175 labelled cells, as measured by the silver removal method. The shaded area represents the expected DNA profile for labelled cells.

2.3.4 Mitotic and Labelling Indices

Mitotic and labelling indices for 2 CAPT cultures were obtained, as described in the Materials and Methods Section, and are recorded in Table 2.1. The counts were made on samples of the FLM analyses.

TABLE 2.1

	Mitotic Index ± S.E.M.	Labelling Index ± S.E.M.
Culture A	0.019 ± 0.022	0.057 ± 0.013
Culture B	0.019 ± 0.0034	0.096 ± 0.004

2.3.5 Cell Cycle Analysis

FLM Analyses:-

Two FLM analyses were performed on separate cultures, each between 24 h and 66 h after innoculation. The cultures, termed A and B were set up on 14/2/78 and 1/5/78 respectively. The resultant FLM curves are shown in Fig. 2.15, and the raw data is presented in Tables 2.2 and 2.3. The durations of G_2, S, and the total cycle time have been determined both by heuristic (eye-fitting) analysis, as explained in the Materials and Methods Section, and by computer analysis (Table 2.4). Estimates of mitotic duration were obtained from scores of mitotic index (see Table 2.1), and the values for G_1 duration were calculated by differences for the heuristic analysis.

For each sample, a 2×2 contingency test (with Yates' correction) has been performed to test the significance of the differences between scores 1 and 2. The χ^2 values, and corresponding probabilities are recorded in Tables 2.2 and 2.3. Taking probabilities of greater than 5% to indicate no significant difference in the scores, it can be seen that for culture A the majority (18 out of 27) of the samples tested fall above the 5% level, and for culture B, an even greater proportion (20 out of 25) fall above this level. The overall χ^2 value has also been computed for each culture, and, although these values both indicate that there is a significant difference between the two sets of data ($P < 0.001$) these high values are due to only a small number of samples with large discrepancies between scores 1 and 2. If these values are discounted, the total χ^2 value for both cultures indicate no significant differences between the two scores (see Tables 2.2 and 2.3).

There are fewer large discrepancies between samples scored for culture B than for culture A. The operator (the author) was the same for all counts on culture B, but was different for the scores 1 and 2 of culture A. Thus the large differences which occur in a few samples of FLM of culture A are probably largely due to differences in operator assessment of "labelled" and "unlabelled" cells. In all cases, slides were randomized prior to counting, to prevent any biased estimations by operators. It was, however, felt to be worthwhile to have one set of counts made by a separate individual, since the scatter of points and FLM value attained in the first peak were possibly due to operator error. However, the separate counts give similar curves; for culture A.

Table 2.4 gives mean cycle phase durations, and variance, plus

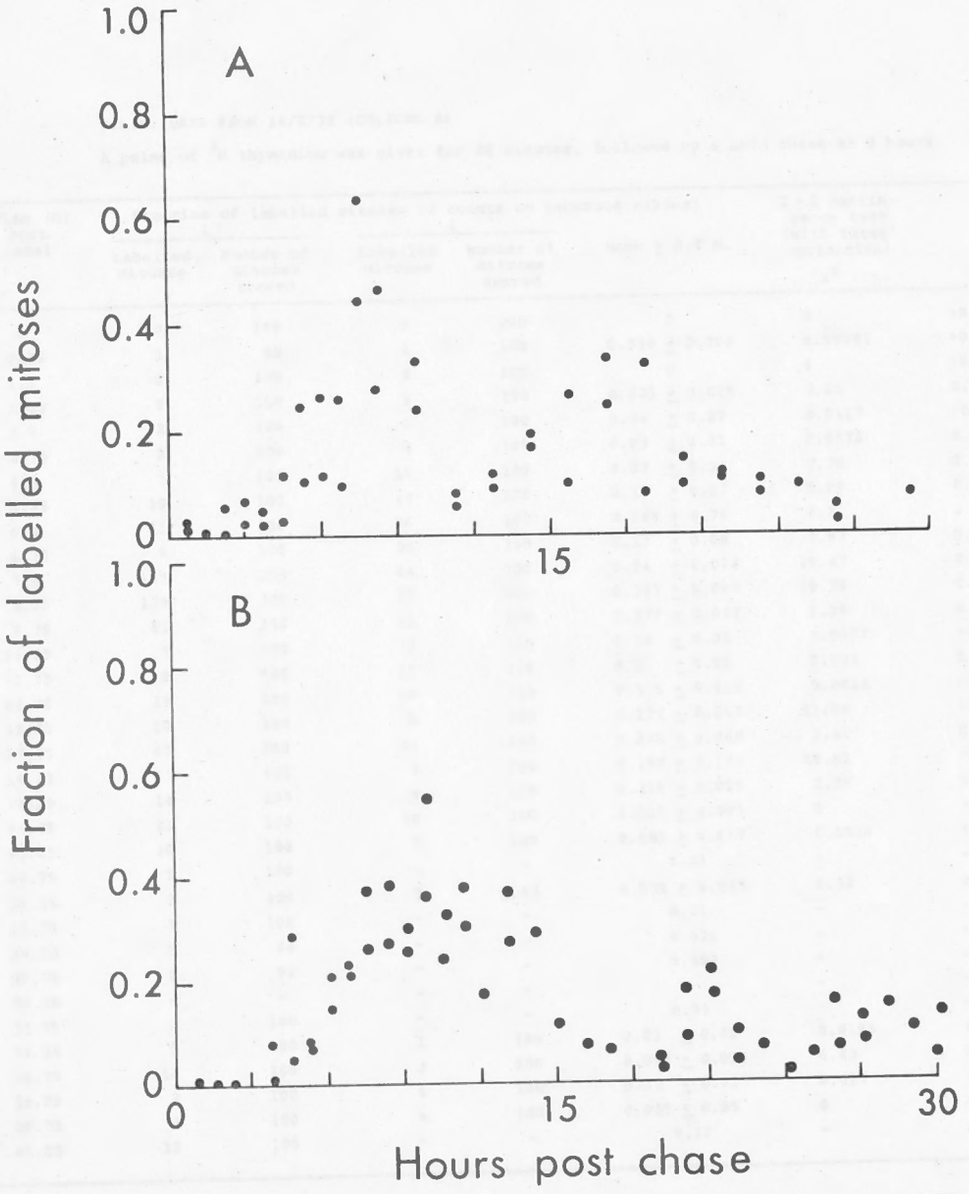


FIG. 2.15 Fraction of labelled mitoses (FLM) curves for two CAPT cell populations.

TABLE 2.2 - F.L.M. DATA FROM 14/2/78 (CULTURE A)

A pulse of ^{3}H thymidine was given for 20 minutes, followed by a cold chase at 0 hours.

Sample number	Time (h) post label	Fraction of labelled mitoses (2 counts on separate slides) 1		2		Mean ± S.E.M.	2 × 2 contingency test (with Yates' correction) χ^2	P
		Labelled mitoses	Number of mitoses scored	Labelled mitoses	Number of mitoses scored			
1	0	0	100	0	100	0	0	>0.9
2	0.75	1	85	2	100	0.016 ± 0.004	0.00081	>0.9
3	1.5	0	100	0	100	0	0	>0.9
4	2.25	0	100	5	100	0.025 ± 0.025	3.28	0.1 to 0.05
5	3.0	2	100	6	100	0.04 ± 0.02	0.0117	>0.9
6	3.75	2	100	4	100	0.03 ± 0.01	0.0172	0.9 to 0.5
7	4.5	3	100	11	100	0.07 ± 0.04	3.76	0.1 to 0.05
8	5.25	10	100	24	100	0.17 ± 0.07	5.99	0.05 to 0.01
9	6.0	11	100	26	100	0.185 ± 0.75	6.5	0.05 to 0.01
10	6.75	9	100	25	100	0.17 ± 0.08	7.97	0.01 to 0.001
11	7.5	159	250	44	100	0.54 ± 0.098	10.47	0.01 to 0.001
12	8.25	139	300	27	100	0.367 ± 0.097	10.76	0.01 to 0.001
13	9.75	81	250	23	100	0.277 ± 0.047	2.59	0.5 to 0.1
14	11.25	5	100	7	100	0.06 ± 0.01	0.0097	>0.9
15	12.75	9	100	11	100	0.1 ± 0.01	0.028	0.9 to 0.5
16	14.25	19	100	16	100	0.175 ± 0.015	0.0014	>0.9
17	15.75	50	190	9	100	0.177 ± 0.087	11.08	<0.001
18	17.25	67	200	24	100	0.288 ± 0.048	2.42	0.5 to 0.1
19	18.75	57	180	7	100	0.194 ± 0.124	20.81	<0.001
20	20.25	14	100	9	100	0.115 ± 0.025	0.79	0.5 to 0.1
21	21.75	11	100	10	100	0.105 ± 0.005	0	<0.001
22	23.25	10	100	7	100	0.085 ± 0.015	0.0026	>0.9
23	24.75	9	100	-	-	0.09	-	-
24	26.25	2	100	3	61	0.035 ± 0.015	0.32	0.9 to 0.5
25	27.75	1	100	-	-	0.01	-	-
26	29.25	5	66	-	-	0.076	-	-
27	30.75	8	92	-	-	0.087	-	-
28	32.25	-	-	-	-	-	-	-
29	33.75	3	100	-	-	0.03	-	-
30	34.25	5	100	1	100	0.03 ± 0.02	0.0155	>0.9
31	36.75	14	100	3	100	0.085 ± 0.055	6.43	0.05 to 0.01
32	38.25	2	100	4	100	0.03 ± 0.01	0.017	>0.9
33	39.75	3	100	4	100	0.035 ± 0.05	0	>0.9
34	41.25	12	100	-	-	0.12	-	-

Note: Estimates for fraction of labelled mitoses were scored by two different people for samples 1 to 23.

Total $\chi^2 = 93.27$; $P < 0.001$ (27 d.f.); Disregarding samples 10, 11, 12, 17, and 19

Total $\chi^2 = 32.18$; $P = 0.1$ to 0.05.

TABLE 2.3 - F.L.M. DATA FROM 1/5/78 (CULTURE B)

A pulse of ^{3}H thymidine was given for 20 minutes, followed by a cold chase at 0 hours.

Sample number	Time (h) post label	Fraction of labelled mitoses (2 counts on separate slides) 1		2		Mean ± S.E.M.	2 × 2 contingency test (with Yates' correction) χ^2	P
		Labelled mitoses	Number of mitoses scored	Labelled mitoses	Number of mitoses scored			
1	0	4	100	-	-	0.02 ± 0.02	-	-
2	0.75	0	100	1	100	0.005 ± 0.005	0	>0.9
3	1.5	0	100	0	100	0	0	>0.9
4	2.25	1	100	0	100	0.005 ± 0.005	0	>0.9
5	3.0	1	100	-	-	0.01	-	-
6	3.75	8	100	1	100	0.045 ± 0.035	4.19	0.05 to 0.01
7	4.5	29	100	5	100	0.17 ± 0.12	18.75	<0.001
8	5.25	8	100	7	100	0.075 ± 0.005	0.00035	>0.9
9	6.0	15	100	21	100	0.18 ± 0.03	0.85	0.5 to 0.1
10	6.75	23	100	21	100	0.22 ± 0.01	0.0029	0.9 to 0.5
11	7.5	17	65	37	100	0.316 ± 0.054	0.0199	0.9 to 0.5
12	8.25	27	100	38	100	0.325 ± 0.055	2.28	0.5 to 0.1
13	9.0	25	100	29	100	0.27 ± 0.02	0.0023	0.9 to 0.5
14	9.75	55	100	36	100	0.46 ± 0.095	6.53	0.05 to 0.01
15	10.5	24	100	32	100	0.28 ± 0.04	1.21	0.5 to 0.1
16	11.25	38	100	30	100	0.34 ± 0.04	1.1	0.5 to 0.1
17	12.0	17	100	-	-	0.17	-	-
18	13.0	37	100	27	100	0.32 ± 0.05	1.86	0.5 to 0.1
19	14.0	29	100	24	83	0.29 ± 0.0005	0.023	0.9 to 0.5
20	15	42	100	11	100	0.265 ± 0.155	23.1	<0.001
21	16	36	100	7	100	0.215 ± 0.145	23.2	<0.001
22	17	6	100	-	-	0.06	-	-
23	18	-	-	-	-	-	-	-
24	19	5	100	6	50	0.04 ± 0.01	1.48	0.5 to 0.1
25	20	18	100	9	100	0.135 ± 0.045	2.74	0.1 to 0.05
26	21	22	100	17	100	0.195 ± 0.025	0.51	0.5 to 0.1
27	22	7	71	4	100	0.069 ± 0.029	1.49	0.5 to 0.1
28	23	7	100	-	-	0.07	-	-
29	24	2	100	-	-	0.02	-	-
30	25	6	100	-	-	0.06	-	-
31	26	16	100	7	100	0.115 ± 0.045	3.14	0.1 to 0.05
32	27	12	100	8	100	0.1 ± 0.02	0.5	0.5 to 0.1
33	28	15	100	-	-	0.15	-	-
34	29	10	100	-	-	0.10	-	-
35	30	5	100	13	100	0.09 ± 0.04	2.99	0.1 to 0.05

Total χ^2 = 95.97 (with 25 d.f.); P = <0.001

Disregarding samples 7, 20 and 21 total χ^2 = 30.9; P = 0.1.

standard errors, for two complete analyses, one after the method of Koschel* and the other following Macdonald* (1970). The method of Macdonald has also been performed assuming that A = 1.5 (where A = mean number of daughter cells to remain proliferative after a mitosis). This was considered important since the large discrepancy between the cell doubling time (37.6 h) and the total cycle time obtained from FLM analysis indicates that there may be a non-cycling fraction in the CAPT populations. Very similar phase durations are, in fact obtained taking A equal to 1.5.

Koschel's analsyis has used scores 2 of both culture A and B, to yield the fitted curves illustrated in Fig. 2.16 a and b. Neither scores 1, or combined scores would yield a completed run for this analysis.

For the curve-fitting analysis of Macdonald mean values obtained by bulking scores 1 and 2 have been used. This gives more weight to the larger sample sizes counted for some scores. Also, 0.33 h has been added to each sample time, since this analysis uses times post initial addition of label. The resultant curves are shown in Fig. 2.17. Goodness of Fit tests indicate significant differences between observed and fitted curves (see Fig. 2.17). This may be expected since large restraints are applied to the data in order to produce a plot which has the characteristics of an FLM curve. Visually, there is generally a close fit between thc observed and expected data points. Some data points have been removed. Specifically, the first sample of culture B (on post label) has too many mitoses to be consistent with the model,

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* These methods were kindly performed by Dr. K. Koschel (The Cancer Institute, Melbourne), and Dr. P. Macdonald (McMaster University, Ontario, Canada).

and all the points after about 20 h in both cultures are much too low. Deleting the 0 h point does not amount to much. Visually, it doesn't appear to be too far from the fitted curve, but the problem is that the program gives too much weight to this kind of discrepancy, and the point has therefore been removed. The data points after 20 h are probably low due to drop-out of some labelled cells into a nonproliferative fraction. In fact, since a random probability factor (termed the transition probability) may cause considerable variation between individuals in the time of initiation of the next round of division it is often better to determine the cell cycle parameters from a single peak FLM curve (Gould, 1977).

Macdonald's method also gives estimated values for growth fraction, as indicated in Tables 2.2 and 2.3.

The Macdonald analysis gives greater values for G_2 duration than the other analyses, and, in fact, this parameter is greater than the total cycle time for culture A. This latter discrepancy can be explained if a "delayed" G_2 is proposed. For instance, the intervention required to set up, label, and cold-chase a culture may cause most cells to delay their next division by a few hours, the cycle subsequently returning to normal.

The high degrec of scatter on both the ascending and descending limbs of the first peaks in Fig. 2.15 may explain why the duration of S-phase, in particular, is extended in the manual analysis (which takes account of all the data on the broad peak) compared with the computer generated durations (which puts more emphasis on the top most point, so

TABLE 2.4 - CELL CYCLE PARAMETERS FOR 2 CAPT CULTURES AS DETERMINED BY FLM ANALYSES (BOTH HEURISTIC AND COMPUTER METHODS) AND BY MAK'S METHOD, TAKING T_c FROM FLM.

Analysis	$T_c \pm$ S.E.M.	Variance	G_1	S±S.E.M.	$G_2 \pm$ S.E.M.	Variance	M	G_1+G_2	Delayed G_2		Growth Fraction
CULTURE A											
Heuristic	10.4		0	3.6	6.2		0.6				
Computer:											
Koschel	9.13±0.2	41.84±0.2	3.17	1.62±0.1	3.94±0.5	5.37±0.17	0.4				
Macdonald (A=2)	8.78±0.34	4.27±1.15	(-1.96)	0.04±0.3			0.71	8.03	9.99±0.38	19.2±3.1	0.32
Macdonald (A=1.5)	8.64±0.33	4.25±1.16	(-1.995)	0.64±0.19			0.66	7.34	9.33±0.26	16.3±2.2	0.29
Mak's Method	9.13±0.2		4.1	0.53	4.3		0.25				
CULTURE B											
Heuristic	14.4		0	6	7.3		1.01				
Computer:											
Koschel	13.11±0.3	185.6±28.6	3.28	1.95±0.1	7.38±0.1		0.4				
Macdonald (A=2)	17.6±1.2	10*	(1.8)	2.7±1.2			1.1	13.8	12.0±1.9	39.1±19.9	0.42
Macdonald (A=1.5)	17.3±1.3	10*	(1.9)	3.0±0.8			1.0	13.3	11.4±1.6	34.2±16.1	0.43
Mak's Method	13.01±0.3		7.2	1.38	4.32		0.37				

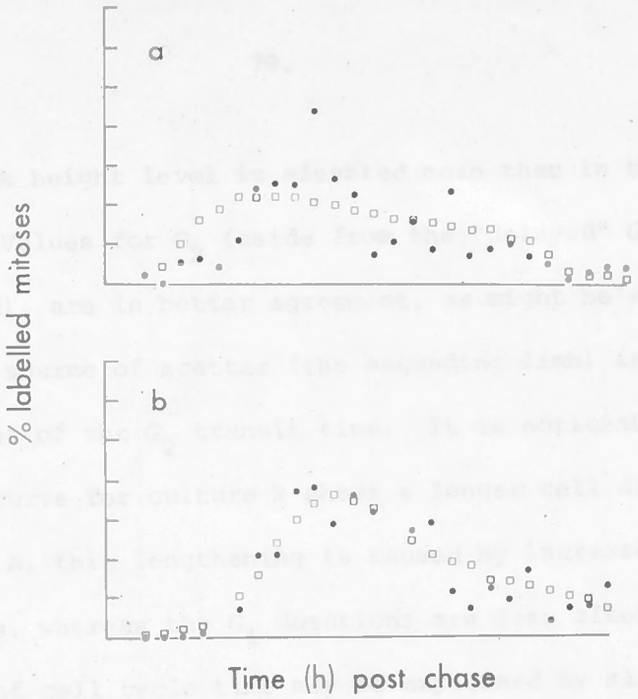


FIG. 2.16 FLM curve fitting analysis of Koschel, for two CAPT cell populations. Dots represent the data points, and the squares represent the fitted line.

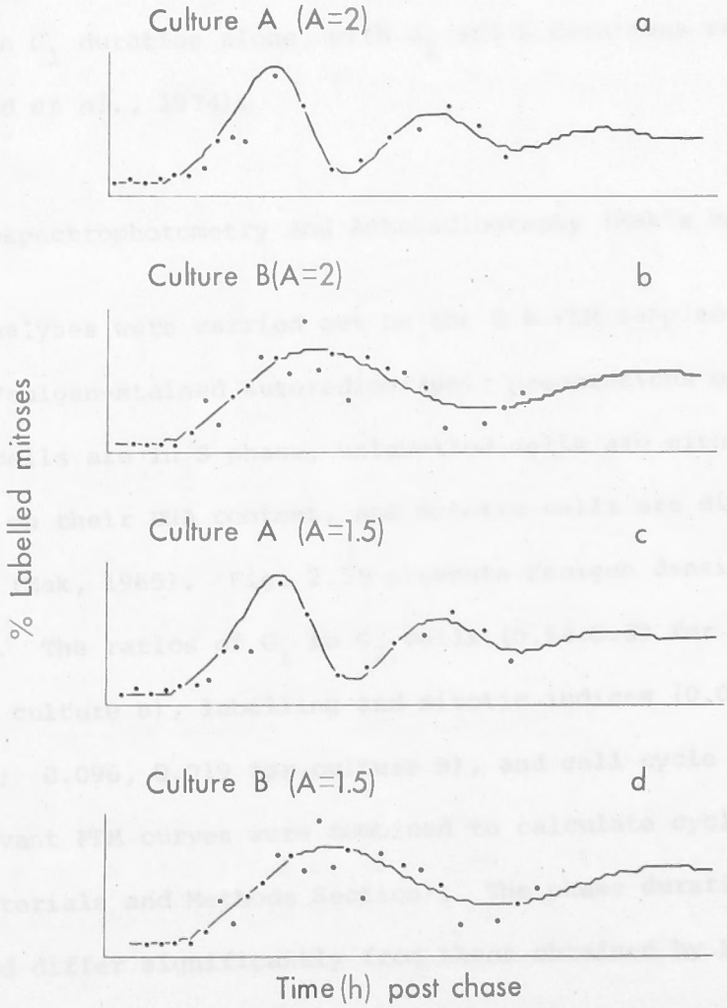


FIG. 2.17 FLM curve fitting analysis of Macdonald, for 2 populations of CAPT (culture A and B), taking A = 1.5 or 2 in each case. Dots represent the data point, and the fitted curves are drawn as solid lines.

that the half peak height level is elevated more than in the intuitive, manual method). Values for G_2 (aside from the "delayed" G_2 durations already explained), are in better agreement, as might be expected, because only one source of scatter (the ascending limb) is involved in the measurement of the G_2 transit time. It is noticeable that whereas the FLM curve for culture B shows a longer cell division time than for culture A, this lengthening is caused by increased duration of S, G_2 and mitosis, whereas the G_1 durations are less altered (Table 2.4). Such elongation of cell cycle time may be explained by slight alterations in culture conditions. However, previous studies with proliferating non-tumorous suspension cultures have indicated that extension of cell division time in different cell cultures of the same cell lines, is due to variation in G_1 duration alone, with G_2 and S durations remaining constant (Gould *et al.*, 1974).

Combined Microspectrophotometry and Autoradiography (Mak's Method):-

These analyses were carried out on the 0 h FLM samples of cultures A and B. In Feulgen-stained autoradiographic preparations of these samples all labelled cells are in S-phase, unlabelled cells are either in G_1 or G_2, depending on their DNA content, and mitotic cells are distinguished cytologically (Mak, 1965). Fig. 2.18 presents Feulgen densitometry data obtained. The ratios of G_1 to G_2 cells (0.54:0.39 for culture A; 0.64:0.26 for culture B), labelling and mitotic indices (0.057,0.019 for culture A; 0.096, 0.019 for culture B), and cell cycle times derived from the relevant FLM curves were combined to calculate cycle phase-durations (Materials and Methods Section). The phase durations determined by this method differ significantly from those obtained by FLM analysis

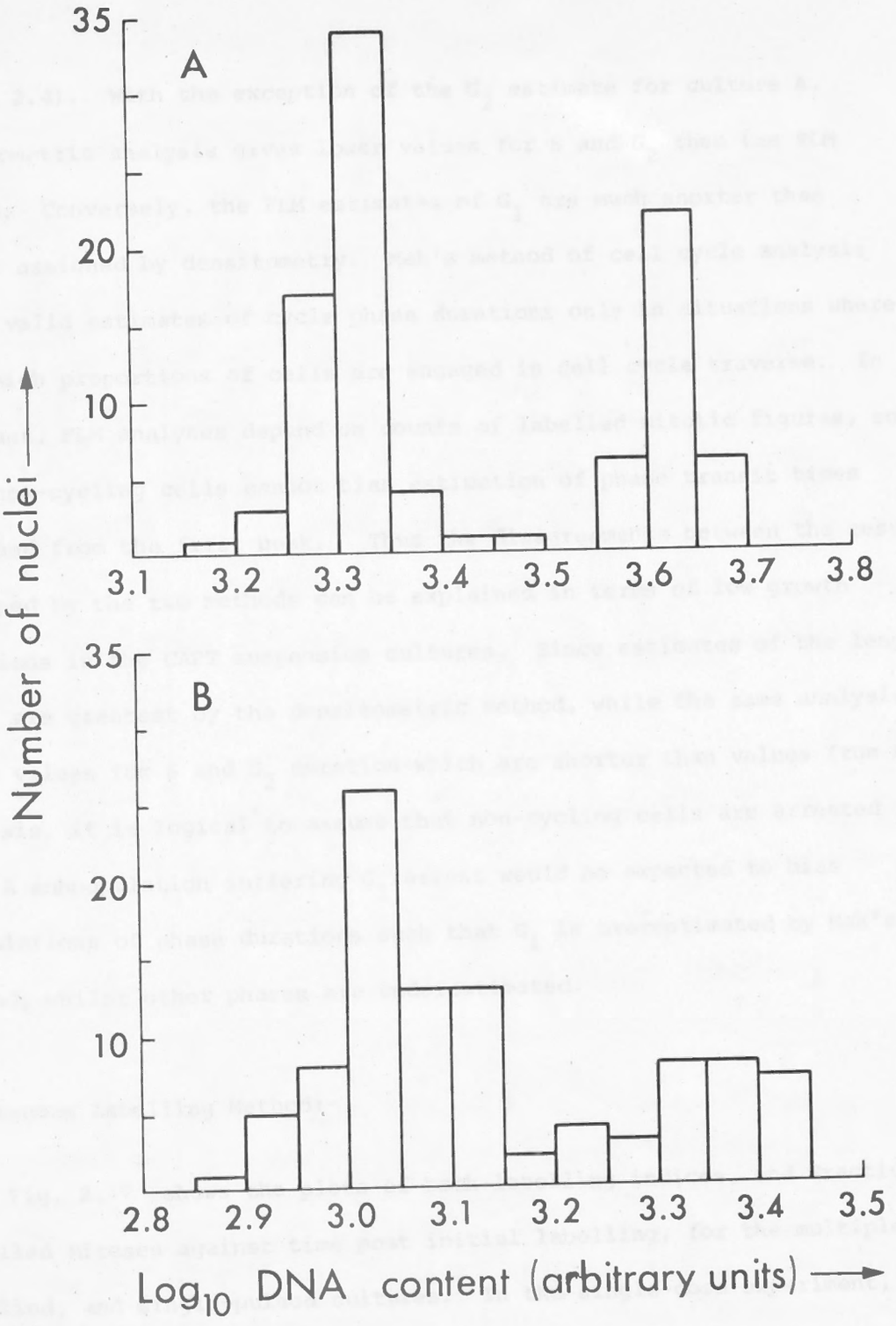


FIG. 2.18 Frequency distributions of values for Feulgen-DNA contents of unlabelled interphase nuclei in 2 rapidly dividing CAPT cultures (cultures A and B 24 h after innoculation). Each distribution represents the staining density of 100 nuclei, which showed no grain (*i.e.* G_1 and G_2 nuclei) in autoradiographs of cells exposed to tritiated thymidine for 20 min. Differences in scale range arise from variation in overall feulgen staining intensity in different staining runs.

(Table 2.4). With the exception of the G_2 estimate for culture A, densitometric analysis gives lower values for S and G_2 then the FLM method. Conversely, the FLM estimates of G_1 are much shorter than values assigned by densitometry. Mak's method of cell cycle analysis gives valid estimates of cycle phase durations only in situations where very high proportions of cells are engaged in cell cycle traverse. In contrast, FLM analyses depend on counts of labelled mitotic figures, so that non-cycling cells cannot bias estimation of phase transit times obtained from the first peak. Thus the disagreements between the results obtained by the two methods can be explained in terms of low growth fractions in the CAPT suspension cultures. Since estimates of the length of G_1 are greatest by the densitometric method, while the same analysis gives values for S and G_2 duration which are shorter than values from FLM analysis, it is logical to assume that non-cycling cells are arrested in G_1. A subpopulation suffering G_1 arrest would be expected to bias calculations of phase durations such that G_1 is overestimated by Mak's method, whilst other phases are underestimated.

Continuous Labelling Method:-

Fig. 2.19 shows the plots of both labelling indices, and Fraction of Labelled Mitoses against time post initial labelling, for the multiple-labelled, and single-pulsed cultures. In the single-dose experiment, the labelling index shows a plateau at approximately 0.16 after 6 h, whilst the fraction of labelled mitoses begins to rise at approximately 4 h and peaks at 0.76 (higher than for the pulse labelled FLM analyses since the cohort of labelled cells is much wider). The G_2 parameter estimated from both single and multiple dose experiments is 6.5 h, which is in good agreement with the value obtained from heuristic analysis of the FLM curves.

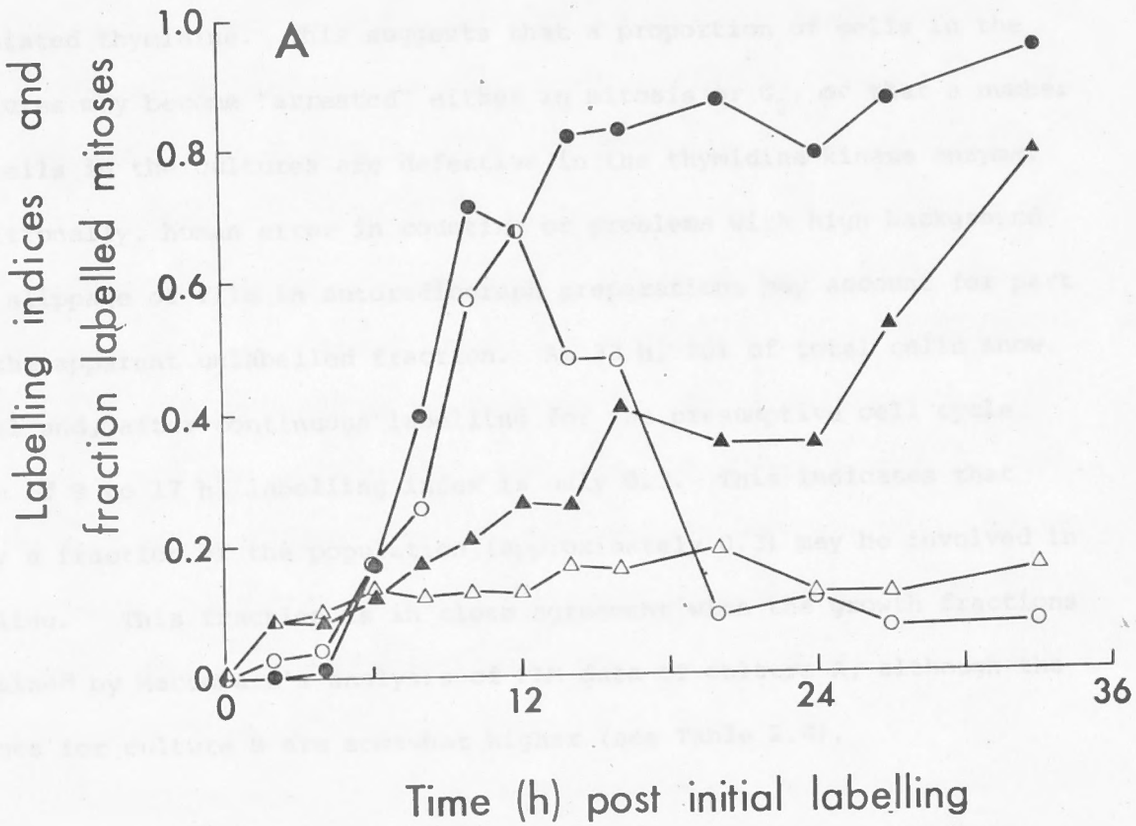


FIG. 2.19 Accumulation of labelled mitoses and total labelled cells in CAPT cell cultures after (i) single dose of tritiated thymidine (1×10^{-8} mol l^{-1}) [——Δ—— labelling index; ——o—— fraction of labelled mitoses], and (ii) multiple doses of tritiated thymidine (1×10^{-8} mol l^{-1} every 6 h) [——▲—— labelling index; —— ● —— fraction of labelled mitoses]. Each data point represents counts on 2,000 nuclei for labelling index, and 100 mitotic figures for determination of fraction of labelled mitoses.

With label continuously available in the multiple dose experiment, the fraction of labelled mitoses reaches a plateau at about 0.8 after 15 h and rises only slowly, thereafter. A small fraction (approximately 5% of mitoses still remain unlabelled even after prolonged supply of tritiated thymidine. This suggests that a proportion of cells in the cultures may become "arrested" either in mitosis or G_2, or that a number of cells in the cultures are defective in the thymidine kinase enzyme. Additionally, human error in counting or problems with high background and slippage of film in autoradiograph preparations may account for part of the apparent unlabelled fraction. At 33 h, 80% of total cells show label and, after continuous labelling for the presumptive cell cycle time of 9 to 17 h, labelling index is only 0.3. This indicates that only a fraction of the population (approximately 0.3) may be involved in cycling. This fraction is in close agreement with the growth fractions obtained by Macdonald's analysis of FLM data of culture A, although the values for culture B are somewhat higher (see Table 2.4).

Extrapolation from this data yields an estimate of total cycle duration of approximately 47 h.

2.4 DISCUSSION

No single cell cycle analysis method, applied in isolation, can give a complete description of cell proliferation kinetics in a population. In this analysis of a tumorous suspension cell culture of *Crepis capillaris*, even analysis by 3 different methods (FLM, Densitometry, Continuous Labelling) does not yield an easily comprehensible model of cell proliferation. Large discrepancies in duration of cell cycle time (cycle time from FLM analysis is 8→17 h, but approximately 47 h from continuous labelling analysis), and

phase durations (G_1 from FLM analysis is close to 0 h, but considerably larger for densitometric analysis) occur. Such discrepancies could be due to limitations of the labelling procedures relied on in all 3 methods. However, care has been taken to assess the accuracy of the methods, and problems caused by accumulation of large thymidine pools in cells of the population, or discontinuity of DNA synthesis in S-phase, have, at least, been discounted (see Results Section 2.3.2 and 2.3.3).

Thus, there seem to be real differences in the apparent cell cycle parameters, as measured by the 3 methods, which highlights the need for multiple approaches to such studies. These discrepancies can, however, readily be explained by proposing a low growth fraction. The presence of a sub-population of non-cycling cells can adversely affect the accuracy of both densitometric and continuous labelling analysis. In the case of the densitometric analysis, all cells have been included in determination of phase durations (Table 2.4), whereas most of the G_1 cells are most probably in a resting state, even in an exponentially dividing population. In the continuous labelling analysis, total cycle time is estimated from the time taken for all cells in the population to be labelled. However, where a low growth fraction exists, some cells may never become labelled, and the apparent cycle duration becomes longer then the "true" value.

The FLM analysis, on the other hand, specifically follows only those cells which are cycling, so that the low growth fraction is not such a severe problem, and the values for cycle parameters can be more readily accepted. However, in the present analysis, the FLM curves are rather flattened, and indistinct, with a scattering of values. This

makes derivation of phase durations difficult.

The low values for the FLM peaks suggest the possibility that 2 sub-populations exist in the CAPT culture, one having a G_2 of approximately 6.5 h and forming the first peak, and the other having a G_2 value of between 14 and 20 h and forming the second peak. However, the FLM peak after a single dose of ^3H Tdr which is not followed by a cold chase reaches a value of 76% at 10 h (Fig. 2.19), and the duration of G_2 estimated from this curve is 6.5 h. Therefore, the majority (80%) of the cells traverse G_2 in this shorter time, and the idea of sub-populations having different G_2 parameters can be discounted.

The FLM analysis is most successful in situations where S-phase is long relative to G_2 and mitosis. In this situation, S-phase cells traverse rapidly into mitosis allowing little asynchrony to develop in the labelled cohort and the relatively narrow mitotic "window" is saturated with labelled cells for an easily measured period (i.e. the breadth of the well defined peak is the duration of S-phase). These conditions apply particularly to the mammalian cell cycle (Quastler and Sherman, 1959; Baserga and Wiebel, 1969). However, in CAPT suspensions the duration of G_2 appears to be long in comparison with S-phase (see Table 2.4). Thus, during the long G_2 stochastic processes may develop asynchrony within the labelled cohort and the mitotic "window" is unlikely to become saturated by the relatively narrow band of S-phase cells. The damped nature of the FLM curves in this analysis can therefore be understood in terms of the temporal structure of the S-G_2-mitosis sequence.

Despite the flattened nature of the FLM curves, the parameters derived

from these curves give the best description of the cell cycle in a population which has a growth fraction of less than 1. The values for total cycle time for all 4 FLM determinations closely agree (Table 2.4), as do mitotic values obtained by both heuristic and the Macdonald computer analysis (Table 2.4). Values for S-phase are considerably shorter for the Macdonald analysis since a "delayed" G_2 is proposed, but, in all cases, S is of short duration. All 4 methods are in agreement as regards the absence, or extremely short duration of G_1 phase.

As a further attempt to verify the shortened cell cycle described by FLM data, the DNA profiles of the labelled cohort of cells have been determined for the "between peak" period of FLM A. If G_1 is indeed short in duration, the labelled cells should enter S-phase fairly rapidly after leaving mitosis. 54 measurements each have been made on 4 samples from FLM of culture A, and the resultant DNA profiles are recorded in Fig. 2.20. Silver grain was removed from the labelled cells prior to measurement of absorbance at 570 nm. It can be seen that, whereas there is an obvious "S-phase" gap in the profiles from 9.75 h and 11.25 h samples, by 12.75 h a considerable fraction of cells has entered S-phase as expected if G_1 is extremely short. By 14.25 h this fraction has decreased, and a larger percentage of cells have the G_2 DNA content. These aspects of the data,then, support the rapid transit of dividing cells from one division to the next. It is also noted, that a considerable number of cells have the G_1 DNA content, suggesting that there may be a real, although reasonably short, G_1 phase in some cells. Additionally, however, the fraction of cells with G_1 DNA content has increased by 12.75 h indicating either that some cells in the population have an extended G_1 phase, or, in support of the idea of non-cycling sub-population, that certain of

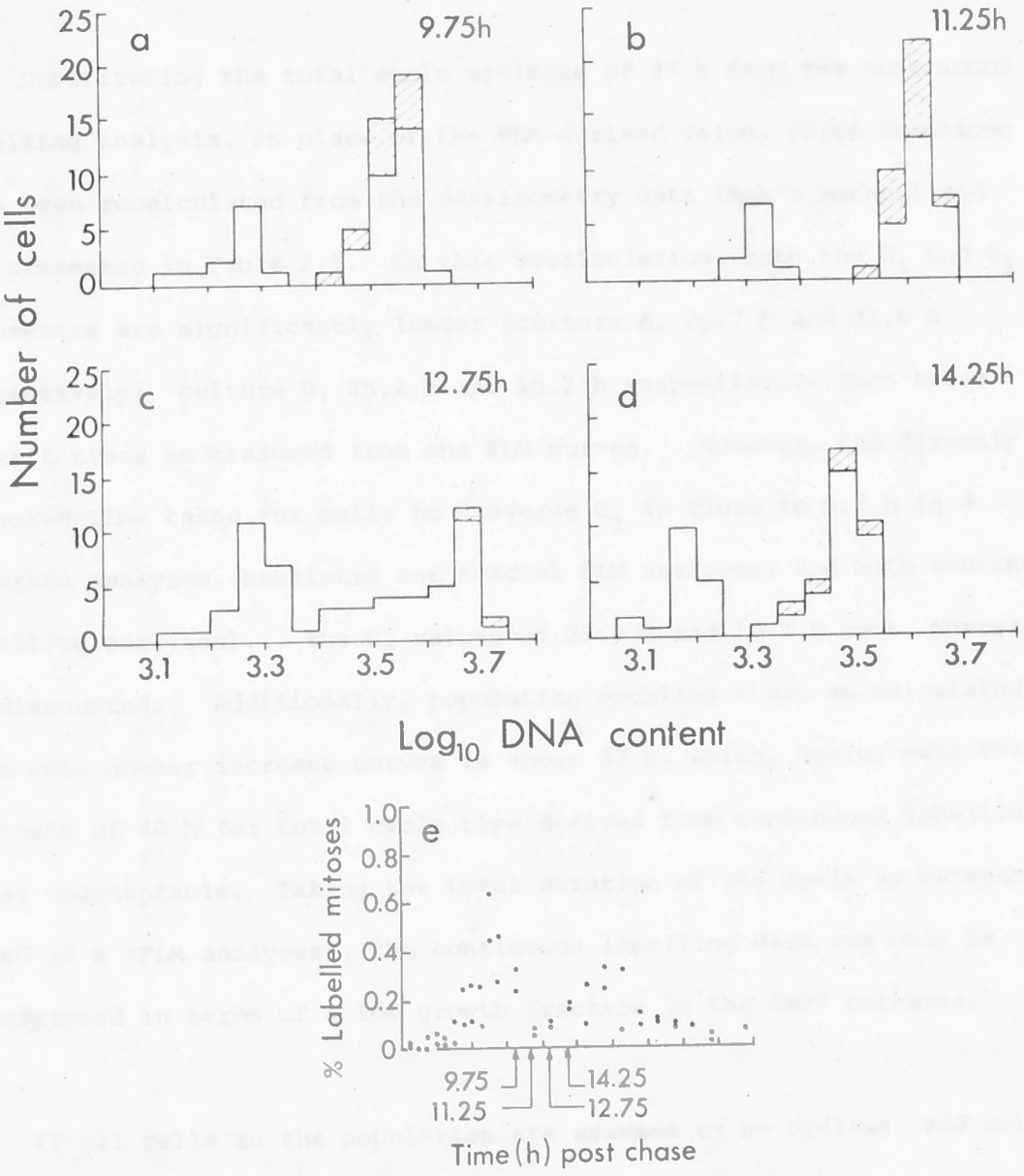


FIG. 2.20 DNA content profiles for 4 samples (9.75 h, 11.25 h, 12.75 h, 14.25 h) taken from the FLM curve of culture A as shown (e). Mitotic figures are represented by shaded columns.

these G_1 cells have, in fact, dropped out of the division cycle at this stage.

Substituting the total cycle estimate of 47 h from the continuous labelling analysis, in place of the FLM-derived value, phase durations have been recalculated from the densitometry data (Mak's Method) and are presented in Table 2.5. In this recalculation, both the G_1 and G_2 parameters are significantly longer (culture A, 20.7 h and 21.6 h respectively; culture B, 25.2 h and 15.2 h respectively) than those transit times as measured from the FLM curves. However, the directly measured time taken for cells to traverse G_2 is close to 6.5 h in 4 separate analyses (heuristic and Koschel FLM analyses, and both continuous labelling analyses). The G_2 values of 21.6 h and 15.2 h must, therefore be discounted. Additionally, population doubling time, as calculated from cell number increase curves is about 37 h, which, again, make the estimate of 46 h for total cycle time derived from continuous labelling data, unacceptable. Taking the total duration of the cycle as between 8 and 17 h (FLM analyses), the continuous labelling data can only be interpreted in terms of a low growth fraction in the CAPT cultures.

If all cells in the population are assumed to be cycling, and cell division time is taken as 12 h (mean of FLM analyses), predictions of increase in cell number with time can be made. Comparison between predicted and observed cell number increases and estimates of the fractions of cells which must therefore leave the cell cycle at each stage of the growth cycle, are presented in Table 2.6. Using this approach, it appears that 0.77 of cells innoculated do not enter the division cycle, and subsequently between 0.2 and 0.4 of the dividing population exit from

TABLE 2.5 - CELL CYCLE PARAMETERS OF 2 CAPT CULTURES, AS DETERMINED USING MAK'S METHOD. VALUE FOR T_c TAKEN FROM 2 DIFFERENT ANALYSES

Type of Analysis	Phase Durations (h)				
	T_c	G_1	S	G_2	M
CULTURE A					
Mak's method, assuming all cells cycle (T_c from continuous labelling)	46	20.7	2.7	21.6	1.2
Mak's method, assuming all G_1 cells are non-cycling and taking T_c from FLM analysis (Koschel)	9.13	0	0.85	7.94	0.53
CULTURE B					
Mak's method, assuming all cells cycle (T_c from continuous labelling)	46	25.2	4.8	15.2	1.3
Mak's method, assuming all G_1 cells are non-cycling and taking T_c from FLM analysis (Koschel)	13.1	0	2.6	9.75	0.98

TABLE 2.6 - ANALYSIS OF CAPT POPULATION ASSUMING CONTINUAL DROP-OUR MODEL

Time Interval (h)	Cell No. Increase (cell $ml^{-1} \times 10^{-5}$)	Fraction of cells assumed to leave division cycle	Fraction of cells in cycling compartment after each interval	Fraction of cells in non-dividing compartment after each interval
0 → 12	4.525 → 5.546	0.774	0.248	0.752
12 → 24	5.546 → 6.918	0.327	0.398	0.602
24 → 36	6.918 → 8.811	0.315	0.427	0.573
36 → 48	8.811 → 10.97	0.423	0.397	0.603
48 → 60	10.97 → 13.87	0.339	0.412	0.587
60 → 72	13.87 → 16.60	0.511	0.339	0.661

the division cycle. A plot has been made of the expected values for fractions of cells labelled during continuous labelling of CAPT suspension cultures between 24 and 72 h of culture (Fig. 2.21). This predicted plot fits quite closely with the experimental plot obtained (Fig. 2.21). Similarly, application of this "continual drop-out" model predicts that 0.6 of the population will be in the non-dividing compartment after 24 h of culture (Table 2.6), which is when the densitometric analyses were carried out. This fraction is very close to the experimentally determined proportion of the population which is in G_1 at this time (Fig. 2.18). Since it has already been argued that non-cycling cells are arrested in G_1 rather than in any other cell cycle phase, it is suggested that the majority of G_1 cells in these cultures is in a resting state. This agrees with data obtained from the FLM curves which indicate that the G_1 phase is either completely absent or at least of short duration, in cycling cells. Recalculation of all phase transit times by applying Mak's method, and excluding all G_1 cells from these estimations, gives values for the lengths of G_2, S and mitosis which are in much closer agreement with the values obtained from the FLM analyses (Table 2.5).

Thus, the "continual drop-out" model which accomodates all of the data and considerations presented above, has the following characteristics:-

(i) Cycling cells have either a very short, or a non-existent G_1 phase.

(ii) In both these tumorous cultures and in root meristems of *Crepis capillaris* the durations of the S, G_2 and mitotic phases are similar (Langridge *et al.*, 1970; Generalova, 1969; Kuroiwa and Tanaka, 1970; Abraham *et al.*, 1968; Van't Hof, 1965) with

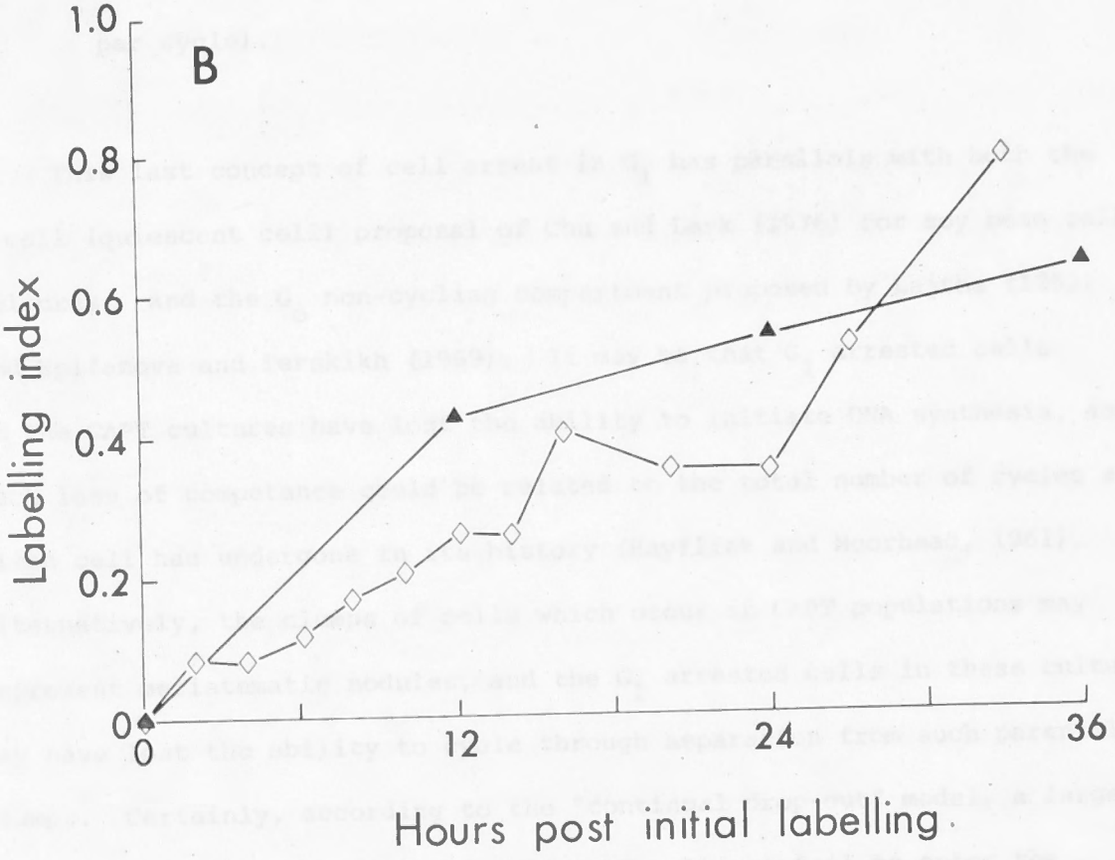


FIG. 2.21 Theoretical (——▲——) and observed (——◇——) accumulation of labelled cells with multiple dose continuously labelled cultures, according to the model proposed in the text.

the possible exception of S-phase, which may be shorter in the cultures. (For CAPT cultures G_1 = 0→2 h; S = 2→4 h; G_2 = 6.5 h; M = 1 h).

(iii) During proliferation in culture a relatively constant proportion of the cycling populations arrest in G_1 (0.3 to 0.4 per cycle).

This last concept of cell arrest in G_1 has parallels with both the Q cell (quiescent cell) proposal of Chu and Lark (1976) for soy bean cell cultures, and the G_o non-cycling compartment proposed by Lajtha (1963) and Epifanova and Terskikh (1969). It may be that G_1 arrested cells in the CAPT cultures have lost the ability to initiate DNA synthesis, and such loss of competance could be related to the total number of cycles a given cell has undergone in its history (Hayflick and Moorhead, 1961). Alternatively, the clumps of cells which occur in CAPT populations may represent meristematic nodules, and the G_1 arrested cells in these cultures may have lost the ability to cycle through separation from such parental clumps. Certainly, according to the "continual drop-out" model, a large fraction of the innoculated cells in CAPT cultures fail to enter the division cycle. However, recently proposed non-deterministic models of cell division control do not require the existence of a non-cycling sub-fraction of the population to explain G_1 "arrested" cells. Rather, such cells are considered still division competent and have not irrevocably left the cell cycle (Shields and Smith, 1977). In terms of the transition probability model (Smith and Martin, 1973), cells in the CAPT suspensions have a probability of initiating a new round of DNA synthesis and division of either close to zero (those cells which become arrested in G_1), or close to one (those cells which traverse G_1 very rapidly).

Previous reports have shown that the plant cell cycle is very much extended in suspension culture as compared with meristematic root tip cells of the same species (Bayliss, 1975) and, in particular, it is the G_1 phase which is increased in length in hormone-requiring suspension cultures (Gould, 1977). However, this analysis of tumorous CAPT suspension cultures indicates a total cycle duration close to that in root tips of the same species, and the G_1 transit time rather than being extended, is of extremely short duration. Thus the possibility arises that although exogenously supplied auxin is essential for growth in non-tumorous and non-habituated plant cell cultures, such growth is achieved only at the expense of an extended G_1 period. Bayliss (1975) has reported a shortened cell cycle time (32.8 h) during embryogenesis in auxin-free suspension cultures of *Daucus carota*, as compared with the normal non-embryogenic cultures to which 2,4-D was supplied (cycle time = 50.9 h). In particular, the G_1 phase was most reduced in the auxin-free culture. Parallels with the CAPT suspension culture, therefore, suggest a link between auxin autotrophy and a shortened cell cycle time, in which the G_1 phase is specifically most reduced in duration. This raises the possibility that the exogenously supplied auxin (2,4-D) is directly inhibiting the rapid transit of cells from G_1 into the DNA synthesis period.

In CAPT cultures the growth fraction is less than one bacause a proportion of the cycling population arrests in G_1. In cycling cells G_1 is very short or absent, and the mean total cycle time of about 12 h is uncharacteristically short for a plant cell suspension culture. These unusual kinetics also feature in animal tumour cell populations, where low growth fractions (not necessarily smaller than in the corresponding normal populations - Gavosto and Pileri, 1971) commonly occur (Mendelsohn,

1962; Baserga, 1965; Bresciani, 1968) as do shortened cycle times (Reiskin and Mendelsohn, 1964; Dormer *et al.*, 1964; Iverson *et al.*, 1962; Bresciani, 1965), and absence of G_1 in the cycling cells (Baserga, 1963; Lala and Patt, 1966). Likewise, dividing CAPT cells that are autotrophic for auxin, with their rapid transit through G_1, are similar to virally-transformed animal cells, which are far less sensitive to reductions in serum levels in terms of proliferation than are their untransformed counterparts. This suggests that a general feature of tumorous cells (both plant and animal) may be the ability to traverse the G_1 phase rapidly and somewhat independently of culture conditions.

This analysis of the cell cycle in the CAPT cell culture has, then, revealed departures from kinetics of proliferation normally found in plant systems. These abnormal features of an extremely short, or absent G_1 phase and a low growth fraction, may be related to the tumorous nature of the population and, hence, the presence of the Ti plasmid. If the Ti plasmid is implicated in generating such gross changes in a population's growth pattern, then care should be taken in the explanation of results of attempts to use this plasmid for genetic modification. The information gained about the cell cycle in the CAPT culture is relevant to any further work on this culture. For instance, a protoplast population released from this culture would be expected to contain some G_1 arrested cells. Such cells may behave differently in uptake or fusion experiments. Similarly, S-phase cells will be expected to be represented in small numbers in the population. Such information is of paramount importance in interpretation of experimental results.

CHAPTER 3

DNA AND LIPOSOME BINDING TO PROTOPLASTS OF CAPT AND NS-1

3.1 INTRODUCTION

The field of plant improvement through genetic manipulation becomes more and more relevant in a world where the increasing population puts stress on the limited plant food supplies. Recently developed techniques in plant tissue culture, particularly those for the isolation and culture of protoplasts (Cocking, 1960), open up new possibilities for such manipulations which could not be achieved through plant breeding methods. Plant breeding presents problems in the form of:-

(i) the length of time required to produce new varieties and,

(ii) the natural incompatibility barriers which restrict crosses to closely related species.

The use of plant protoplasts should overcome both of these obstacles. In fact fusion of plant protoplasts is not only possible between widely separated species or genera (*e.g.* between *Petunia hybrida* and *Parthenocissus tricuspidata* (Power *et al.*, 1975) or between *Glycine max* and *Nicotiana glauca* (Kao, 1977)), but higher plant/algal (*e.g.* carrot + Chlamydamonas, Fowke *et al.*, 1979), higher plant/yeast (Davey and Power, 1975), and even inter-kingdom fusions (*e.g.* between human cells and *Haplopappus gracilis* protoplasts (Lima-de-Faria *et al.*, 1977)) have been achieved. Fusion between protoplasts necessarily produces hybrid cells in which the entire genomes of the parent cells are present. For more specific introductions of new genetic material into host cells, isolated DNA or chromosomes, hopefully carrying well defined genetic markers, should be the vectors of choice. Criticisms which have been directed at many early attempts at DNA uptake into plants centre around:-

(i) the use of intact plant material, which precludes analysis at the single cell level, and

(ii) the use of material which is not free from bacterial contamination. The use of protoplasts from sterile tissue cultures would circumvent both of these problems. The lack of a cell wall on protoplasts also reduces the barrier to the entry of genetic material in the form of isolated DNA, nuclei and organelles, all of which have now been successfully introduced into plant protoplasts by simple techniques (Ohyama *et al.*, 1972; Potrykus and Hoffmann, 1973).

Attempts to monitor the fate of such exogenously supplied genetic material may be more readily achieved in low chromosome number species, and this chapter presents results of an investigation into possibilities for DNA uptake into one of these plant species (*C.capillaris*). The successful introduction of foreign genetic material into cells includes the uptake of the material, its integration and/or replication, its expression in the recipient cell and its preservation in the progeny. However, the work reported here is restricted to characterising the binding of DNA to protoplasts since this is the first step in such a process. Previous work with other species has often by-passed this initial stage and merely monitored the fate of the foreign material once it is inside the cell. Such monitoring has most often relied on biochemical techniques (Ohyama *et al.*, 1978) but in the present study a cytological approach has been chosen since this allows more direct observation of DNA/protoplast interaction. The additional use of quantitative autoradiography and Feulgen microspectrophotometry allows assessment of both the amount of DNA binding (given by the quantity of silver grains, since tritium labelled DNA is used), and also the DNA content and therefore the cell cycle stage of the "host" protoplasts. Such cell cycle related information is of interest since the idea of "competence" for transformation at certain stages of the growth

cycle, which has been demonstrated with transformable bacteria (Hotchkiss, 1954) is a concept which has not been addressed in higher plant transformation studies. In one experiment, protoplasts released from plant cells of varying ages have been shown to differ in levels of exonuclease activity (Ohyama *et al.*, 1978), indicating that cell age may indeed be an important factor for DNA uptake into plant cells.

Liposomes are being increasingly used for the introduction of biologically active macromolecules into mammalian cells (Poste *et al.*, 1976). Techniques have been developed for the entrapment of both RNA (Dimitriadis, 1978), and DNA (Papahadjopoulos, 1978) into lipid vesicles. It is possible to incorporate such genetic material into cells using liposomes as vehicles, since fusion of liposomes with the cell membrane allows release of the vesicle contents into the cell. These methods have not been applied to plant material but this should be readily achieved with the use of protoplasts. A study has therefore been made of the binding of small unilamellar liposomes to both CAPT and NS-1 protoplasts. Protoplasts of NS-1 have been used in this study, for comparative purposes, as an example of a normal, non-tumorous plant population, as opposed to the tumorous cells of the CAPT culture. As with the DNA binding experiments, a cytological approach has been used, and the relationship between cell cycle stage and binding capacity has similarly been investigated. Liposomes of varying phospholipid compositions have been employed since the type of liposome has been shown to be important for successful fusion of liposomes with mammalian cells (Papahadjopoulos *et al.*, 1973).

In both the DNA and liposome binding experiments described here tests

have been made on the extent of binding in the presence of varying amounts of the polycation poly-L-ornithine. Several previous workers have noted the marked enhancement of DNA uptake by plant protoplasts in the presence of polycations (*e.g.* Ohyama *et al.*, 1972).

The surface properties of protoplasts will be important for the binding of both DNA and liposomes and for sound theoretical reasons these properties may be expected to vary with cell cycle stage. To maintain membrane composition exactly stable throughout the cycle every single membrane component would have to be synthesised and/or integrated into the membrane absolutely synchronously. Such "tight-linkage" between the cell cycle and membrane synthesis is extremely unlikely (Mitchison, 1971) and therefore fluctuations in membrane constitution may reasonably be expected. In mammalian cells, one such property, that of overall surface charge as measured by electrophoresis, is different in mitotic cells as compared with interphase cells (Brent and Ferrester, 1967). Similar studies with plant protoplasts used leaf tissue in which all cells are presumably in the G_1 state and, as expected, have constant overall surface charge within a species, as measured by electrophoretic mobility (Nagata and Melchers, 1978). It is known from these studies that the overall surface charge on both animal cells and plant protoplasts is always negative and Nagata and Melchers (1978) discuss the implications of this charge in relation to cell-cell adhesion and the binding of tobacco mosaic virus (TMV) to protoplasts. They suggest that the use of polycations such as poly-L-ornithine for uptake of TMV gives a positive coating to the virus particles allowing them to adhere to the otherwise negatively charged protoplast surface. It is possible to separate a heterogeneous population of cells into sub-populations according to cell surface properties, by countercurrent

distribution using a two-phase polymer system (Walter *et al.*, 1971), and one of the main determinants of the partition of cells in such systems may be their surface charge. This technique has therefore been utilised here to look for cell cycle related differences in surface properties, in NS-1 protoplasts, which may then relate to differential binding of DNA or liposomes.

Since protoplasts rather than whole cells are being used in these investigations, the effect of the protoplast isolation procedure on the cell cycle has been investigated. In particular, the enzyme treatment may inhibit progression through the cell cycle so that protoplasts may be in a non-cycling state when binding experiments are carried out. Alternatively, the isolation procedure may preferentially release protoplasts of particular cell cycle stages. The previous analysis of the cell cycle in the CAPT culture (see Chapter 2) is very useful in these binding studies. For example, it is now known that the majority of G_1 cells in the culture are in a non-cycling state, and this may affect the degree of DNA binding achieved. Even if the G_1 cells do have a greater binding capacity, this may be of little interest since the induction of division in these protoplasts may not be possible. It is also known that S-phase is of very short duration in the CAPT culture so that protoplasts in S-phase may represent a very small fraction of the population analysed. Any differential response of S-phase cells may thus go unnoticed in such a population and experiments have been designed to cope with this problem.

3.2 MATERIALS AND METHODS

3.2.1 Cell Cycle Studies on Protoplasts

Protoplasts were isolated from suspension cell cultures by the techniques described in the General Materials and Methods Section. For cell cycle studies part of the culture was pre-labelled with tritiated thymidine, prior to protoplast isolation, whilst the remaining cells were left unlabelled and samples were removed and flash labelled at intervals during protoplast isolation and culture.

Pre-labelling:-

CAPT cells were "pulse-chase" labelled with tritiated thymidine as described in the Materials and Methods Section of Chapter 2. A small sample was removed and fixed in 3:1 ethanol:acetic acid (this becoming the sample pre 0 h), whilst the remaining cells were incubated with wall-removing enzyme for protoplast isolation. A further sample was removed, during enzyme treatment, at 5.5 h (pre 5.5 h). After 12 h protoplasts were separated from cell clumps, washed free of enzyme, placed in a special protoplast culture medium (see Table 3.1) and incubated, in the dark, at 27°C, on a slowly rotating plate. Samples were removed and fixed at 12 h, 17 h and 32 h (*i.e.* after 0 h, 5 h, and 20 h of culturing).

Post-labelling:-

A small sample of a population of unlabelled CAPT cells was removed and fixed prior to addition of enzyme to the remaining cells for protoplast isolation. This sample is denoted "post 0 h". A further sample was removed from the enzyme mixture at 5.5 h (post 5.5 h), flash labelled with tritiated

TABLE 3.1 - PROTOPLAST CULTURE MEDIUM

B5 salts, iron and vitamins
1000 mg/L casamino acid
3 mM $CaCl_2.2H_2O$
2 mg/L 2, 4-D
0.5 mg/L kinetin

thymidine by the method described in Chapter 2, and fixed in 3:1 ethanol: acetic acid. Protoplasts were washed free of enzyme after 12 h and placed in protoplast culture medium as previously described for the pre-labelled calls. Samples were removed, flash labelled and fixed at 12 h, 17 h, and 33 h (*i.e.* after 0 h, 5 h, and 21 h of culturing).

All samples, from both pre and post-labelling procedures were Feulgen stained and autoradiographs were made. Labelling and mitotic indices were calculated where appropriate, and DNA-content profiles of labelled protoplasts were produced by microspectrophotometry for labelled protoplasts of the pre-treatment method.

An additional study was undertaken using leaf tissue of *Crepis capillaris*, to determine the cell cycle state of cells within this tissue (by DNA content) both before and after protoplast isolation. Leaf protoplasts were isolated as described in General Materials and Methods Section.

3.2.2 Preparation of Labelled CAPT DNA

CAPT cultures were pulsed with tritiated thymidine at 10^{-7} mol l^{-1} (specific activity, 5 Ci m mol^{-1} [185 GBq m mol^{-1}], which gives an activity

of 0.5 μ Ci ml^{-1} [18.5 kBq ml^{-1}]), each day of the 7 day culture period between innoculations into fresh media. At the end of the week, cells were harvested and DNA was isolated by a modified method of Heyn *et al.* (1974). DNA was stored in a freezer and thawed for each use.

3.2.3 Liposome Techniques

3.2.3.1 Liposome Preparation

Preparation of unlabelled liposomes:-

Small unilamellar liposomes were prepared by mixing the required phospholipids in 5 ml of Protoplast Washing Buffer (13% sorbitol; 3 mM $CaCl_2$; 2 mM Na H_2PO_4) in a hand-held homogeniser. A phospholipid concentration of 7 μ moles/ml was used, which should yield approximately 10^{15} vesicles per ml. The resultant suspension was homogenised thoroughly and then sonicated, using a MSE 100 watt Ultrasonic Disintegrator, for approximately 10 min, or until a translucent suspension was produced. Throughout all these steps, the suspension was kept cool with ice. The surface charge on liposomes produced in this way partly depends on the phospholipid composition. The 3 phospholipids used in this study are L-α-Phosphatidyl choline (Sigma, 100 mg/ml in 9:1 chloroform:methanol, also known as L-α-Lecithin), which is a neutral phospholipid, L-α-Phosphatidyl-L-serine (Sigma, 10 mg/ml in 95:5 chloroform: methanol) which is a negatively charged phospholipid, and stearylamine (Sigma, powder form), which is a positively charged phospholipid. Liposomes with neutral, negative, and positive charge were all prepared using the following phospholipid compositions:-

(a) Neutral liposomes:- 100% phosphatidyl choline (5.25 mg/ml).

(b) Negatively charged liposomes:- 90% phosphatidyl choline (4.725 mg/ml) + 10% phosphatidyl serine (0.525 mg/ml).

(c) Positively charged liposomes:- 90% phosphatidyl choline (4.725 mg/ml) + 10% stearylamine (0.19 mg/ml).

The stearylamine was first dissolved in 9:1 chloroform:methanol, and, in the preparation of all 3 liposome types the phospholipid mixtures were dried down on to the bottom of the homogeniser prior to the addition of PWB. Drying was achieved by evaporation of the chloroform/methanol mixture in a flow of nitrogen gas.

The shape and size range of the produced liposomes was investigated using electron microscopy, as described later.

Preparation of labelled liposomes:-

For the use of liposomes in autoradiographic studies tritiated cholesterol was added to the phospholipid mixtures. The label was used at 0.2×10^{-3} m mol L^{-1} (specific activity 9.5 Ci m mol^{-1} [352 GBq m mol^{-1}], Radiochemical Centre Amersham), which gives an activity per ml of 2 µ Ci (74 k Bq ml^{-1}). 100 µl of a 1 in 10 dilution of the triated cholesterol stock was added to each liposome preparation.

3.2.3.2 Liposome Analysis

Microscopic examination of liposomes:-

Liposome samples were studied under the electron microscope. A drop of liposome preparation was first placed on a carbon-coated grid, left for approximately 1 min, and then stained for a further minute with a drop of 2% (w/v) sodium phosphotungstate. The grid was then dried with filter paper and examined under a Hitachi H-500 electron microscope.

Distribution of labelled cholesterol in liposomes:-

Labelled liposomes of all 3 phospholipid compositions were prepared as described. A much lower level of sorbitol (50 mM) was used in the buffer since 13% sorbitol will not float on the ficoll gradient which is used. Each liposome preparation (1 ml) was gently added to a stepwise ficoll gradient, previously prepared in a polyallomer ultracentrifuge tube. The gradient was composed of 2 ml each of 20%, 10%, 8%, 5% and 3% ficoll dissolved in buffer (50 mM sucrose; 5 mM tricine; 0.5 mM EDTA; pH 7.5), and carefully layered on top of one another in the tube. The tubes, complete with sample, were then spun in an ultracentrifuge at 35,000 rpm for 17 h, in a SW41 rotor. After centrifugation each tube was pierced at the base, and eleven 1 ml samples were collected. From each original liposome sample, 11 fractions were thus obtained, 50 µl of each fraction was added to 5 ml scintillation fluid (containing 1 ml solubiliser) ready for scintillation counting. The tritium gate on the scintillation counter was set at 80 → 6000 to cut out any large counts at the lower end of the tritium curve which are due to interferance by ficoll.

To estimate the lipid content of each fraction, optical density readings at 260 nm were recorded. For each fraction, comparison was made with the equivalent fraction obtained from a blank tube (*i.e.* ficoll gradient with no lipid sample added), since ficoll also absorbs at 260 nm. 20 µl SDS per ml was added to clear each fraction, so that false readings were not obtained due to cloudiness.

This method gives an indication of:-

(i) the level of incorporation of ^{3}H cholesterol into liposomes.

(ii) the distribution of incorporated ^{3}H cholesterol.

3.2.4 Binding of DNA and Liposomes to Protoplasts

Protoplasts of both the CAPT and NS-1 suspension cell cultures were isolated and washed several times in PWB to remove the wall-degrading enzymes. Estimates of protoplast number per ml were made using a haemocytometer, and viability was determined using Evans Blue stain.

DNA Binding

100 µl of labelled CAPT DNA was added to 3 ml CAPT protoplasts in a 10 ml tube, and gently agitated by rolling the tube between both hands for a few seconds. The agitation process was repeated every minute during a 10 min incubation period, and the protoplasts were then pelleted by centrifugation for 5 min in a bench centrifuge with a swing-out rotor. The pellet was resuspended in 3:1 ethanol:acetic acid containing 13% sorbitol. The sorbitol fixative was replaced, a few hours later, with a fixative containing no sorbitol, and protoplasts were stored at 4°C ready for Feulgen staining and the preparation of autoradiographs, at a later date.

Poly-L-ornithine (at 200 µg/ml, 20 µg/ml and 3 µg/ml) was mixed with the protoplasts prior to addition of DNA. A poly-L-orthine stock (3 mg/10 ml) was freshly made up in water just before it was required.

In some cases, flash labelled protoplasts were used for DNA binding. Labelling (with tritiated thymidine at 10^{-8} mol l^{-1}) was either performed on cells before protoplast isolation, or on isolated protoplasts just prior to DNA addition. Some protoplasts were incubated in culture medium for a few hours before flash labelling and used in DNA binding procedures. Labelling techniques are employed to allow S-phase cells to be readily distinguished in autoradiographs.

Liposome Binding

For liposome binding, 3 ml samples of both CAPT and NS-1 protoplasts were used, as for DNA binding, and agitation and fixation procedures were the same as above. All 3 liposome types (neutral, negatively charged, and positively charged) were used, and either 0.1 ml or 0.5 ml added to each 3 ml protoplast sample. Poly-L-ornithine was used at 20 µg/ml and 3 µg/ml, and protoplast labelling procedures were employed as before.

3.2.5 Aqueous 2-polymer Phase Systems for Partition of Protoplasts

This method which was applied to NS-1 protoplasts, relies on the differential partition of protoplasts between 2 immiscible phases. In this case, aqueous dextran (D), polyethylene glycol (PEG) 2-phase systems have been used. When these polymers are mixed in concentrations above a "critical concentration", such a 2-phase system is produced, whereas below this concentration a homogeneous solution would be obtained. The phase diagram in Fig. 3.1 illustrates the critical point for a Dextran T500, PEG 6000 system. At all points above the curved line (the binodial), separation of the polymers into 2 phases occurs. At different polymer concentrations, shown by open circles in Fig. 3.1, the polymer compositions in the top and bottom phases are given by the junctions of the straight line, (the tie-line) through the point, with the binodial. The top phase has a higher concentration of PEG and lower concentration of D, whereas the reverse holds for the bottom phase.

The partition of cells between the phases depends on the relative affinities of the membrane surface to the polymers constituting the top and bottom phases, and to the cell's adsorption at the interface.

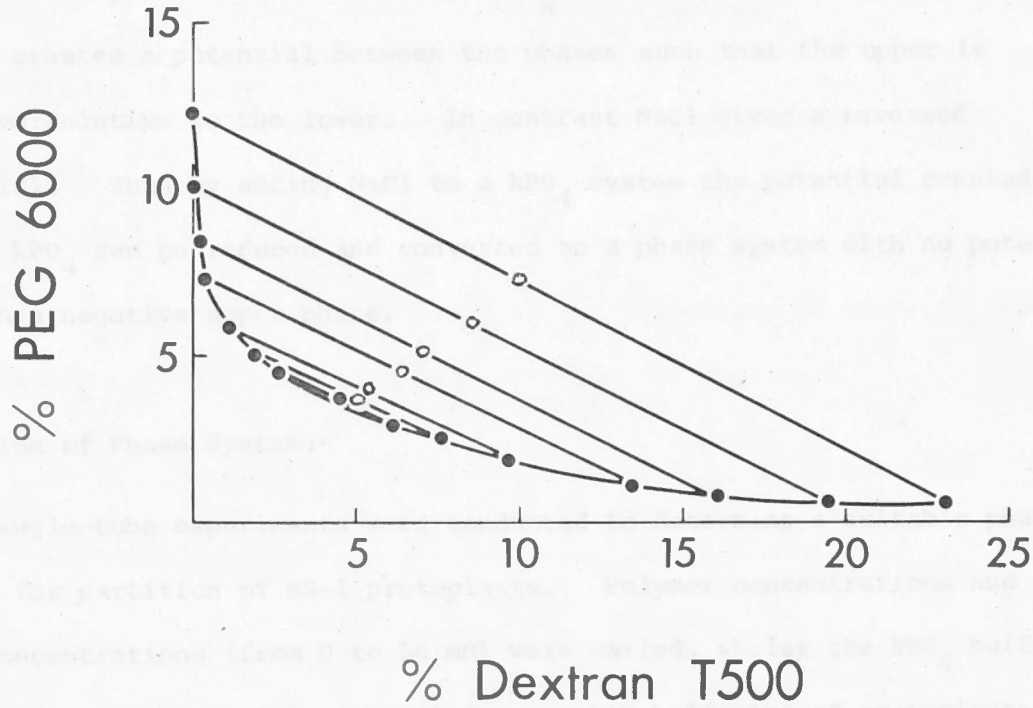


FIG. 3.1 Binodial of the PEG 6000, Dextran T500 system.

The addition of appropriate salts to the system can produce an electrostatic potential difference between the phases, by unequal distribution of the salts between the 2 phases (Reitherman *et al.*, 1973). Such a charge differentiation between the phases increases the separation of cell types which differ in terms of surface charge. The extent of the potential difference produced depends on the particular salt used, the concentration of the salt, and the distance of the phase system from the critical point. In this study, kPO_4 buffer used in every phase system creates a potential between the phases such that the upper is positive relative to the lower. In contrast NaCl gives a reversed potential. Thus by adding NaCl to a kPO_4 system the potential created by the kPO_4 can be reduced and converted to a phase system with no potential or even a negative upper phase.

Selection of Phase System:-

Single-tube experiments were conducted to determine a suitable phase system for partition of NS-1 protoplasts. Polymer concentrations and NaCl concentrations (from 0 to 50 mM) were varied, whilst the kPO_4 buffer at pH 7.8 (containing 13% sorbitol for osmotic buffering of protoplasts) was kept constant at 10 mM. The effect of temperature, and molecular weight of PEG (either 4000 or 6000) on partition, were also studied. Stock solutions of the polymers were prepared, as follows, and stored at 4°C.

(a) Dextran:- Dextran T500 (Pharmacia Fine Chemicals, average molecular weight 500,000) is hygroscopic, so that a 20% (w/w) solution was prepared by weighing approximately 220 g D, and making it up to 1 kg with water. The exact concentration was determined by polarimetry.

(b) Polyethylene Glycol:- Both PEG 4000 and 6000 (from Union Carbide) were used, and 40% (w/w) stock solutions were prepared.

For each single-tube experiment, a total phase system of 3.2 g (quantities for phase systems are usually determined by weight) was prepared from stock solutions. 0.8 ml protoplasts (at approximately 10^5/ml) were added to each tube to made a 4 g system and the capped tubes were then inverted 30 times and left to settle for 15 mins.

Once separation was achieved, 1.5 ml of top phase was removed, diluted with 1.5 ml phosphate buffer, mixed, and the optical density at 440 nm was determined. 2.5 ml buffer was added to the bottom phase and mixed before O.D. determination. Top and bottom phase volumes were determined by weight.

The partition ratio is expressed as the percentage of the total cells in the top phase. Calculations were performed as follows:-

$$\text{Amount in Top Phase} = A^T \times \text{dilution factor (2)} \times \text{top phase volume}$$

$$\text{Total Amount} = \Sigma\ X + Y$$

$$\text{Where } X = A^T \times 3\ (1.5\text{ ml} + 1.5\text{ ml})$$

$$Y = A^B \times (2.5\text{ ml} + \text{bottom phase volume})$$

$$\%\text{ in top phase} = \frac{A^T \times 2 \times \text{top phase volume}}{\Sigma\ X + Y} \times 100$$

A^T = absorbance reading of top phase.

A^B = absorbance reading of bottom phase.

Countercurrent Distribution:-

For more efficient partition of protoplasts, the method of countercurrent distribution, which is a multistage procedure, was used. The principle of counter current distribution is described in many books and reviews *e.g.* Craig (1960). The thin-layer apparatus (previously described by Albertsson,

1971), consists of 2 plexiglas plates with 120 concentric cavities. The bottom plate, which has 60 cavities of 0.7 ml capacity, is fixed to a platform which has a driving motor for shaking, and the top plate, which also contains 60 cavities (which are open at the top to permit loading and emptying of phase system and sample), is free to rotate above this. A tightly fitting cover is placed over the top plate. By rotation of the top plate relative to the lower plate, each cavity of the upper plate can successively be brought into coincidence with each cavity of the lower plate. A diagrammatic representation of the apparatus is shown in Fig. 3.2.

The selected phase system (6.3% D T500, 6.3% PEG 4000, 10 mM kPO_3 pH 7.8, 13% sorbitol, 10 mM NaCl) was prepared, and allowed to settle overnight. 0.6 ml of the bottom phase was added to each bottom cavity of the partition block, and 0.6 ml of the top phase was added to each of the top cavities. Cavities 0 and 30 were left empty. To 6 g of the remaining phase system, 1.6 ml protoplasts (at approximately 2×10^5/ml) in kPO_4 buffer with 13% sorbitol, were added and mixed. 1.2 ml of this mixture were added to each of cavities 0 and 30. By adding samples to both of these cavities, and performing only 30 rotations, 2 distributions were obtained. In this study rotation was performed manually since an automatic device, as described by Albertsson, was not available. 10 min after addition of the samples, the partition block was shaken for 30 sec and left to settle for 8 min. The top plate was then rotated so that each top phase was transferred to the next bottom cavity, and the whole block was again shaken for 30 sec and left to settle for a further 8 min before moving to the next chamber. 30 transfers were performed in this way and the temperature was maintained at 20°C ($\pm$1°C) throughout the experimental period. After

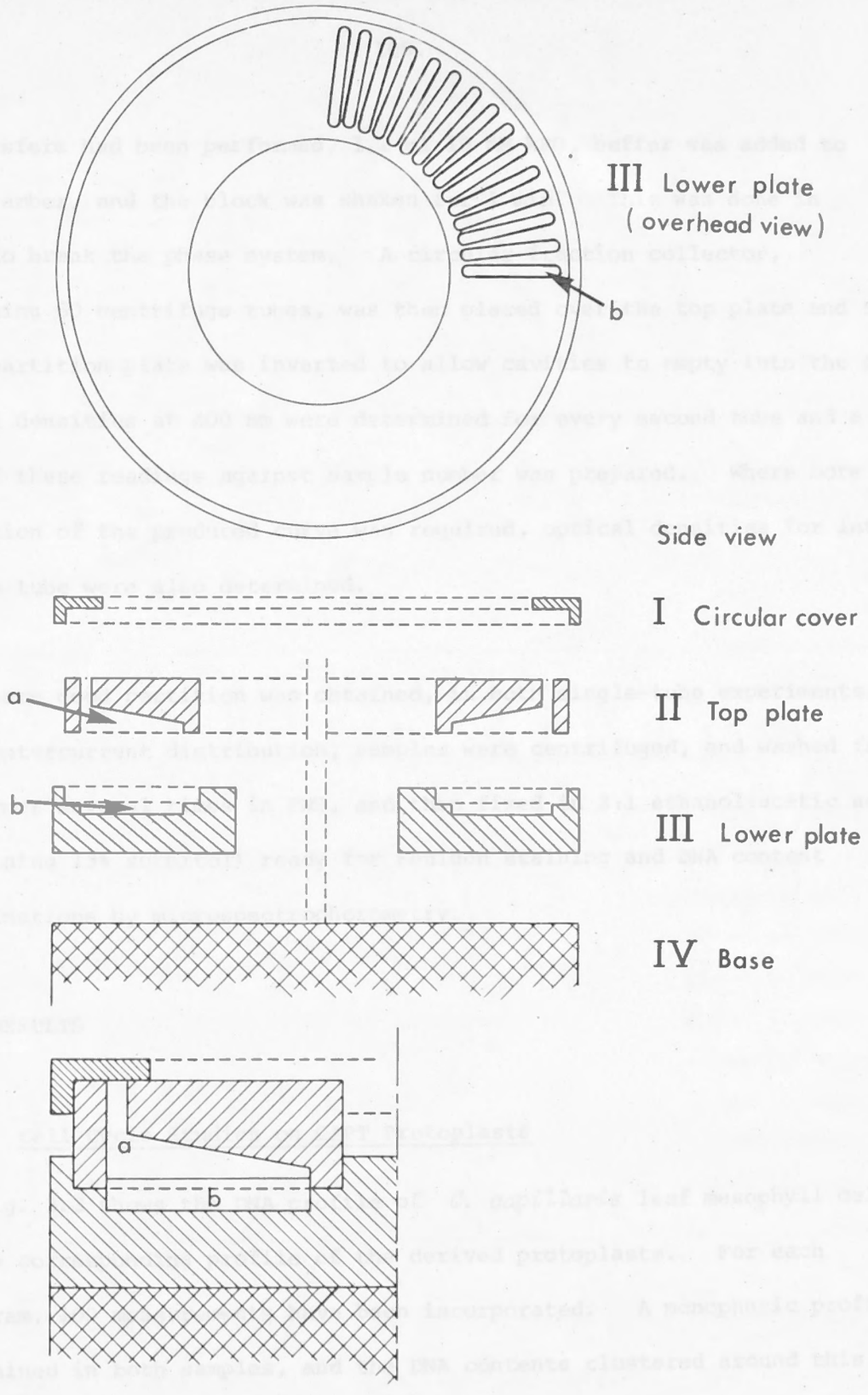


FIG. 3.2 Diagram of the countercurrent distribution apparatus. a = upper cavity, which contains the top phase, and is open at the top to facilitate filling and emptying. b = lower cavity which contains the bottom phase (2 mm depth).

By turning the upper plate relative to the lower plate each cavity of the rotor can be successively brought into coincidence with each cavity of the lower plate. A circular sequence of partition cells is thus formed.

30 transfers had been performed, 1.2 ml 10 mM kPO_4 buffer was added to each chamber, and the block was shaken for 1 min. This was done in order to break the phase system. A circular fraction collector, containing 60 centrifuge tubes, was then placed over the top plate and the whole partition plate was inverted to allow cavities to empty into the tubes. Optical densities at 400 nm were determined for every second tube and a plot of these readings against sample number was prepared. Where more definition of the produced curve was required, optical densities for intermediate tube were also determined.

Where good partition was obtained, in both single-tube experiments, and for countercurrent distribution, samples were centrifuged, and washed free of polymer several times in PWB, and then fixed in 3:1 ethanol:acetic acid (containing 13% sorbitol) ready for Feulgen staining and DNA content determinations by microspectrophotometry.

3.3 RESULTS

3.3.1 Cell Cycle Studies on CAPT Protoplasts

Fig. 3.3 shows the DNA profile of *C. capillaris* leaf mesophyll cells, and the corresponding profile of the derived protoplasts. For each histogram, 100 measurements have been incorporated. A monophasic profile is obtained in both samples, and the DNA contents clustered around this single peak are most closely equivalent in value to G_1 DNA contents observed previously in CAPT cells (see densitometric data in Chapter 2). Thus, cells, in the leaf tissue seem to be arrested in the G_1 state, and the derived protoplasts are consequently all in G_1.

Results of an experiment using pre and post labelling techniques (described in the Materials and Methods Section) to study the cell cycle in CAPT protoplasts, are presented here. DNA profiles of pre-labelled cells and protoplasts are recorded in Fig. 3.4, and labelling and mitotic indices for both pre-labelled and post-labelled cells and protoplasts are recorded in Table 3.2, and the labelling indices have also been plotted (Fig. 3.5).

TABLE 3.2

Sample	Mean Labelling Index ± S.E.M.	Mean Mitotic Index ± S.E.M.
Pre 0 h	0.065 ± 0.001	0.058 ± 0.011
Pre 5.5 h	0.057 ± 0.02	-
Pre 12 h	0.163 ± 0.004	close to zero
Pre 17 h	0.182 ± 0.003	close to zero
Pre 32 h	0.156 ± 0.001	close to zero
Post 0 h	not yet labelled	0.059 ± 0.007
Post 5.5 h	0	close to zero
Post 12 h	0.027 ± 0.01	close to zero
Post 17 h	0.03 ± 0.007	close to zero
Post 33 h	0.0065 ± 0.0008	close to zero

Fig. 3.4a shows the DNA profile of CAPT cells prior to protoplast isolation. Cells of all cycle stages are represented. The DNA contents of flash labelled cells, however, are clustered into one peak of values between the G_1 and G_2 peaks (see Fig. 3.4b). The DNA contents of the labelled population is followed during enzyme isolation and culture, as shown in Figs. 3.4 c→f. At 5 h, whilst the cells are still in enzyme, the DNA content profile is similar to the 0h sample, indicating that no

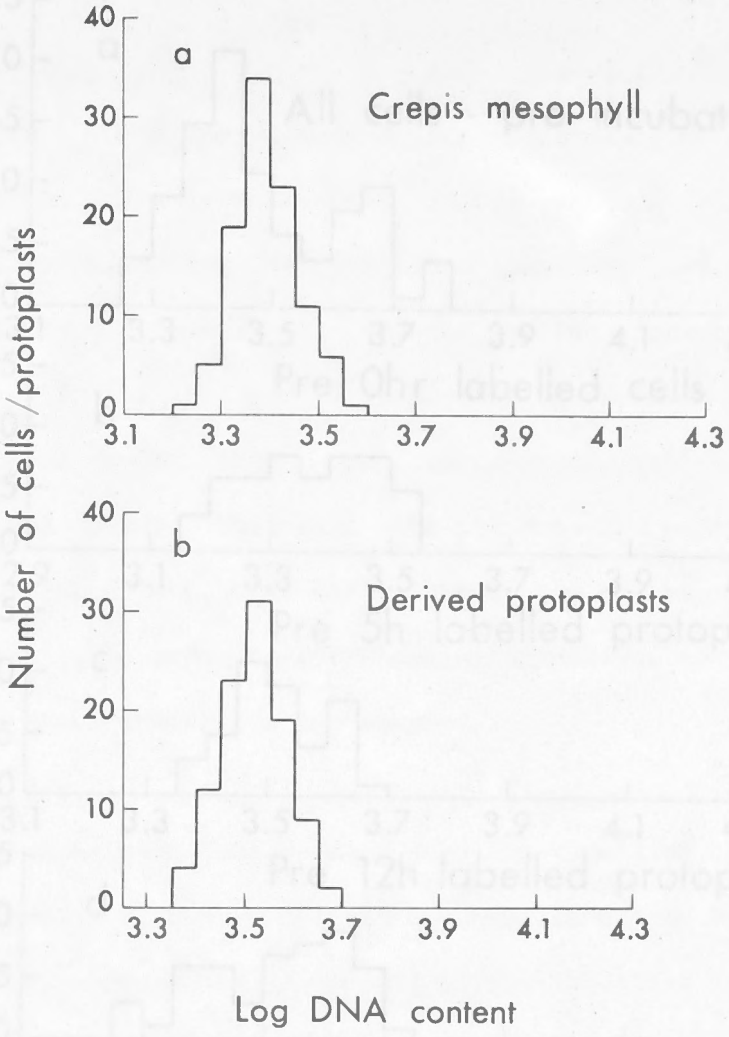


FIG. 3.3 DNA profiles (100 measurements each) of *Crepis capillaris* leaf mesophyll cells (a), and their derived protoplasts (b).

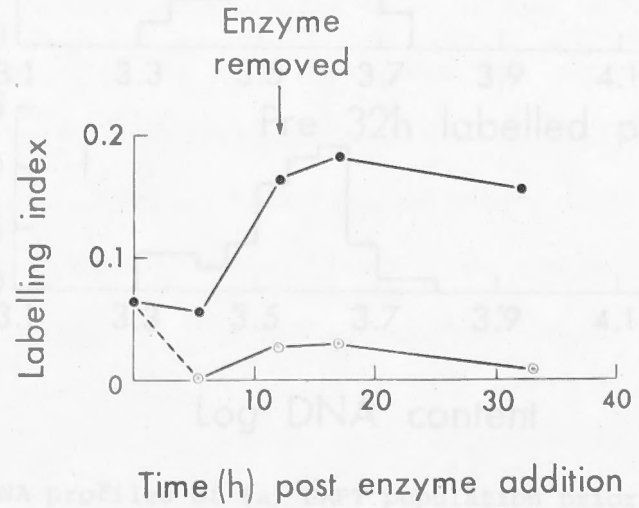


FIG. 3.5 Labelling indices of protoplasts at various times during culturing (*i.e.* after enzyme removal). — ■ — cells were pulse-chase labelled for 20 min prior to protoplast isolation. — ⊙ — protoplasts were pulse-chase labelled for 20 min immediately after enzyme removal.

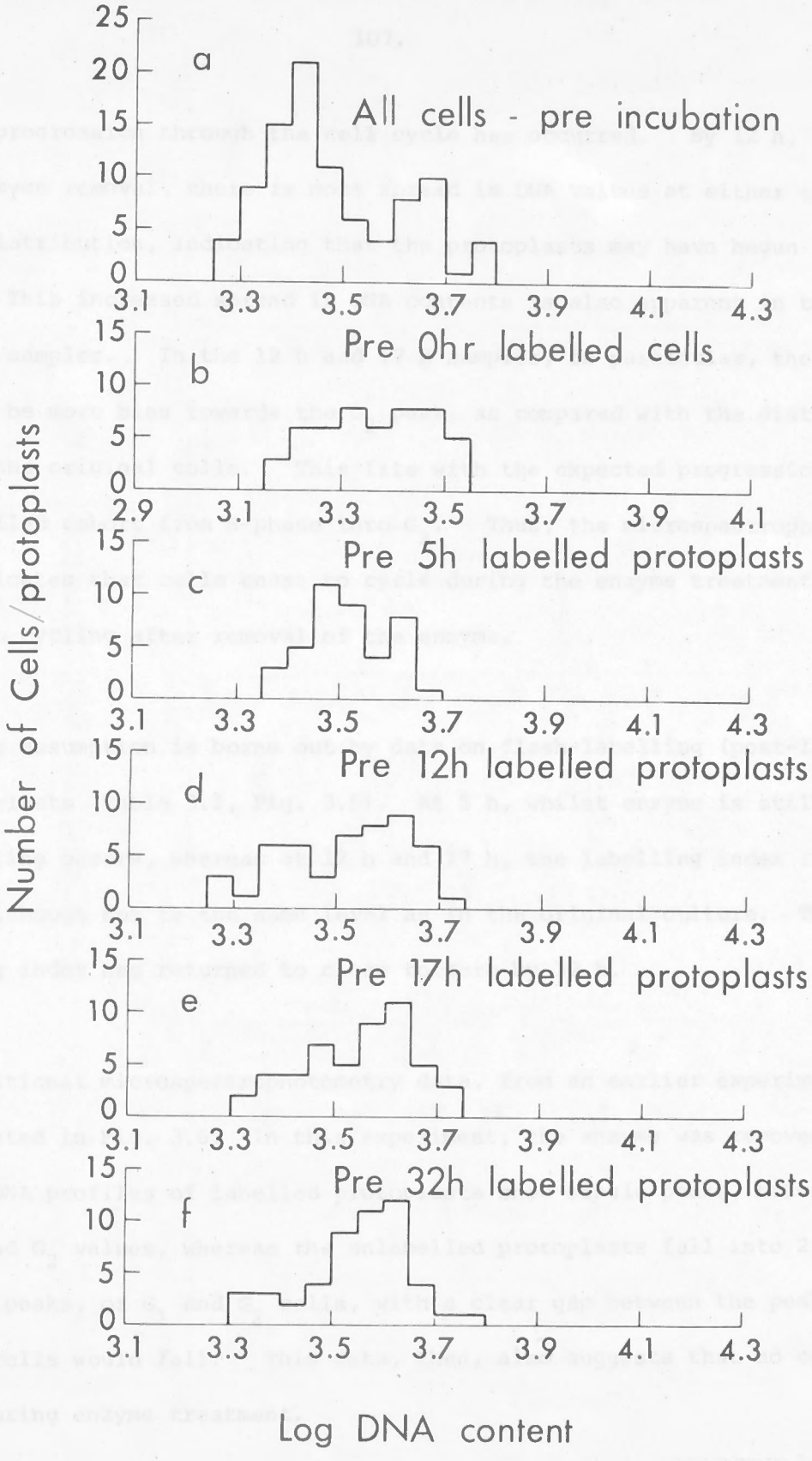


FIG. 3.4 DNA profiles of (a) CAPT population prior to protoplast isolation (100 measurements), (b) labelled population prior to protoplast isolation (50 measurements), (c) labelled released protoplasts after 5 h in enzyme *i.e.* during incubation (42 measurements), (d) labelled protoplasts immediately after enzyme removal (50 measurements), (e) labelled protoplasts after 5 h culture (50 measurements), (f) labelled protoplasts after 20 h culture (50 measurements).

obvious progression through the cell cycle has occurred. By 12 h, just after enzyme removal, there is more spread in DNA values at either end of the distribution, indicating that the protoplasts may have begun to cycle again. This increased spread in DNA contents is also apparent in the 17 h and 32 h samples. In the 12 h and 17 h samples, in particular, there seems to be more bias towards the G_2 peak, as compared with the distribution seen in the original cells. This fits with the expected progression of the labelled cohort from S-phase into G_2. Thus, the microspectrophotometry data indicates that cells cease to cycle during the enzyme treatment, but return to cycling after removal of the enzyme.

This assumption is borne out by data on flash-labelling (post-labelling) of protoplasts (Table 3.2, Fig. 3.5). At 5 h, whilst enzyme is still present, no labelling occurs, whereas at 12 h and 17 h, the labelling index rises again, although not to the same level as in the original culture. The labelling index has returned to close to zero by 32 h.

Additional microspectrophotometry data, from an earlier experiment, is presented in Fig. 3.6. In this experiment, the enzyme was removed at 15 h. DNA profiles of labelled protoplasts show single peaks, between the G_1 and G_2 values, whereas the unlabelled protoplasts fall into 2 distinct peaks, of G_1 and G_2 cells, with a clear gap between the peaks where S-phase cells would fall. This data, then, also suggests that no cycling occurs during enzyme treatment.

It can be seen, from Fig. 3.5 that the labelling indices of isolated protoplasts from pre-labelled cells (*i.e.* from 12 h in graph) are considerably larger (greater than 2 x) than the original labelling index in CAPT

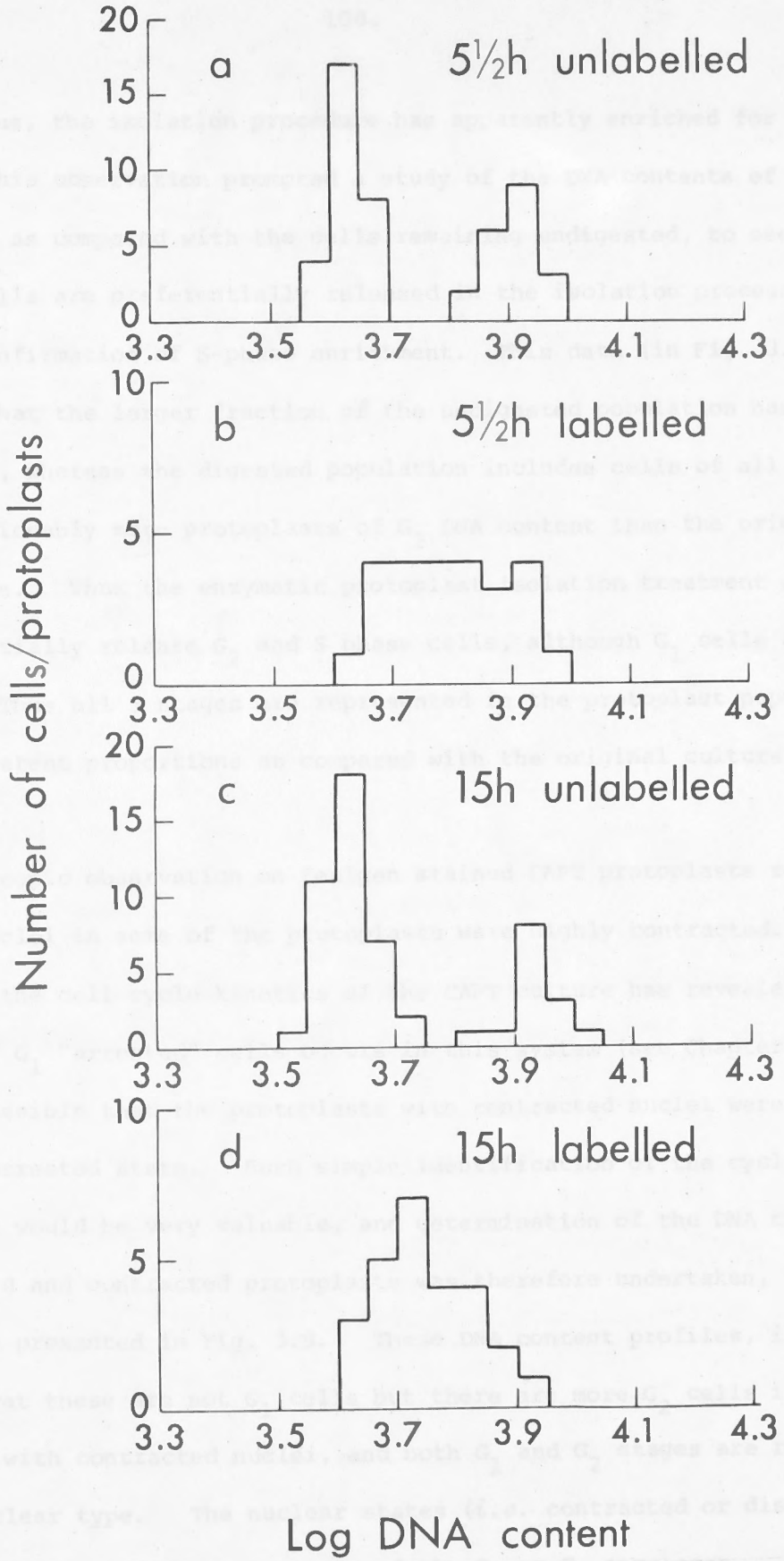


FIG. 3.6 DNA profiles of CAPT cells/protoplasts from a pulse-chase labelled population. (a) Unlabelled released protoplasts after 5.5 h in enzyme (*i.e.* during isolation). (b) Labelled released protoplasts after 5.5 h in enzyme. (c) Unlabelled protoplasts immediately after enzyme removal. (d) Labelled protoplasts immediately after enzyme removal.

cells. Thus, the isolation procedure has apparently enriched for labelled cells. This observation prompted a study of the DNA contents of isolated protoplasts as compared with the cells remaining undigested, to see if either G_1 or G_2 cells are preferentially released in the isolation process, and to look for confirmation of S-phase enrichment. This data (in Fig. 3.7) indicates that the larger fraction of the undigested population has the G_1 DNA content, whereas the digested population includes cells of all 3 phases, and has noticeably more protoplasts of G_2 DNA content than the original CAPT culture. Thus the enzymatic protoplast isolation treatment appears to preferentially release G_2 and S phase cells, although G_1 cells are also released. Thus all 3 stages are represented in the protoplast population, but in different proportions as compared with the original culture.

Microscopic observation on Feulgen stained CAPT protoplasts revealed that the nuclei in some of the protoplasts were highly contracted. Previous studies on the cell cycle kinetics of the CAPT culture has revealed that a fraction of G_1 "arrested" cells occurs in this system (see Chapter 2). It was thus possible that the protoplasts with contracted nuclei were in fact in the G_1 arrested state. Such simple identification of the cycle state of these cells would be very valuable, and determination of the DNA contents of dispersed and contracted protoplasts was therefore undertaken, and the results are presented in Fig. 3.8. These DNA content profiles, in fact, indicate that these are not G_1 cells but there are more G_2 cells in the population with contracted nuclei, and both G_1 and G_2 stages are represented in each nuclear type. The nuclear states (*i.e.* contracted or dispersed) could correspond to different stages of the G_1 or G_2 sequences.

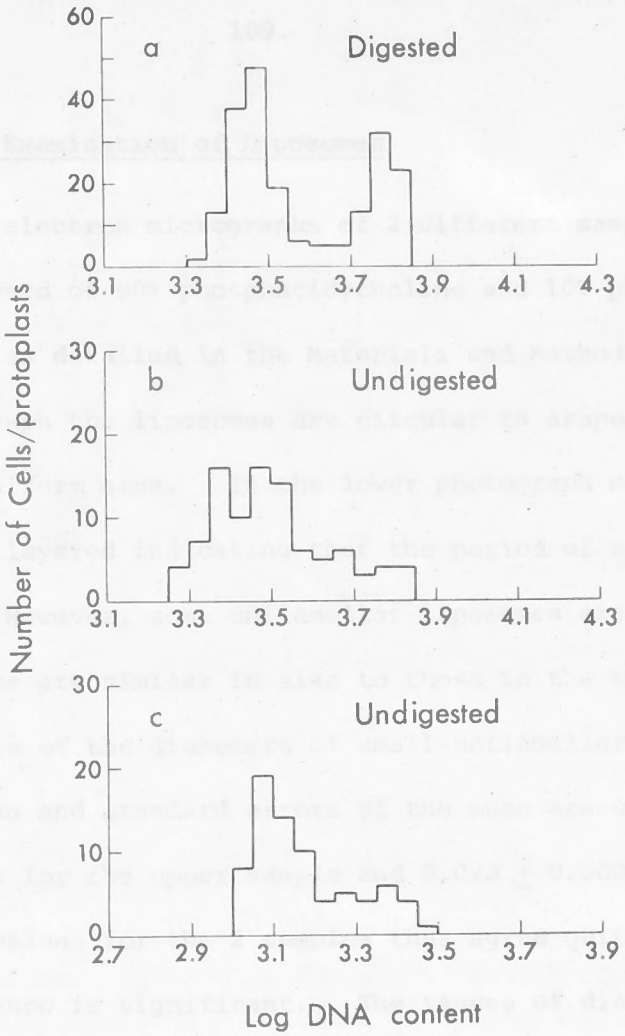


FIG. 3.7 DNA profiles of (a) digested CAPT protoplasts (200), and (b) undigested cells (100) from the same sample.

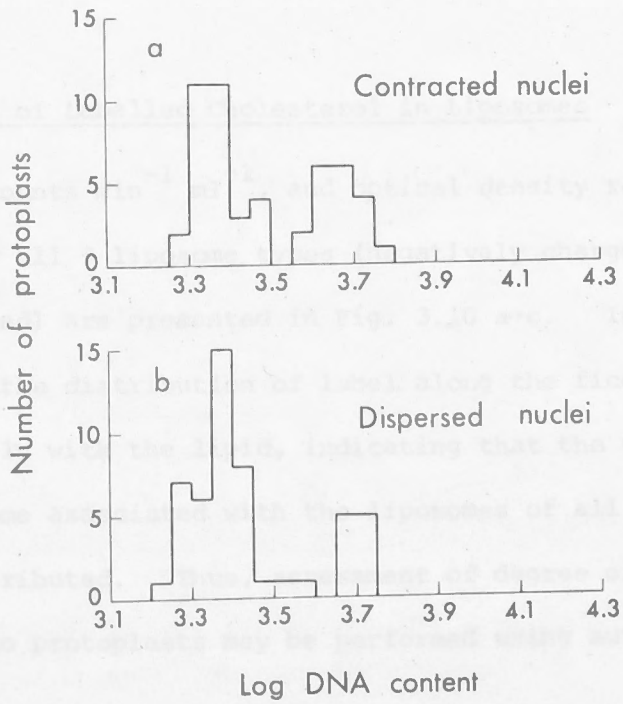


FIG. 3.8 DNA profiles of (a) contracted nuclei (50) of CAPT protoplasts immediately after enzyme removal, and (b) dispersed nuclei (50) from the same sample.

3.3.2 Microscopic Examination of Liposomes

Fig. 3.9 shows electron micrographs of 2 different samples of liposomes both composed of 90% phosphatidycholine and 10% phosphatidyl serine and prepared as detailed in the Materials and Methods Section. In the upper photograph the liposomes are circular in shape as expected, and are of fairly uniform size. In the lower photograph many of the structures are multilayered indicating that the period of sonication was insufficient. However, some unilamellar liposomes are also seen in this sample and these are similar in size to those in the upper photograph. From 100 measurements of the diameters of small unilamellar liposomes in each sample, the mean and standard errors of the mean are calculated to be 0.027 ± 0.00068 μ for the upper sample and 0.023 ± 0.00042 μ for the lower sample. The values for the 2 samples thus agree quite closely, although the difference is significant. The ranges of diameter size are $0.015 \rightarrow 0.046$ μ for the upper sample and $0.014 \rightarrow 0.033$ μ in the lower sample. The mean values are within the range determined by Ryrie (1975) in unilamellar phospholipid vesicles, *i.e.* between $0.02 \rightarrow 0.15$ μ.

3.3.3 Distribution of Labelled Cholesterol in Liposomes

Plots of both counts min^{-1} ml^{-1}, and optical density readings against fraction number, for all 3 liposome types (negatively charged, neutral, and positively charged) are presented in Fig. 3.10 a→c. In all 3 plots it can be seen that the distribution of label along the ficoll gradient coincides very closely with the lipid, indicating that the tritiated cholesterol has become associated with the liposomes of all 3 types, and is quite evenly distributed. Thus, assessment of degree of binding of labelled liposomes to protoplasts may be performed using autoradiography.

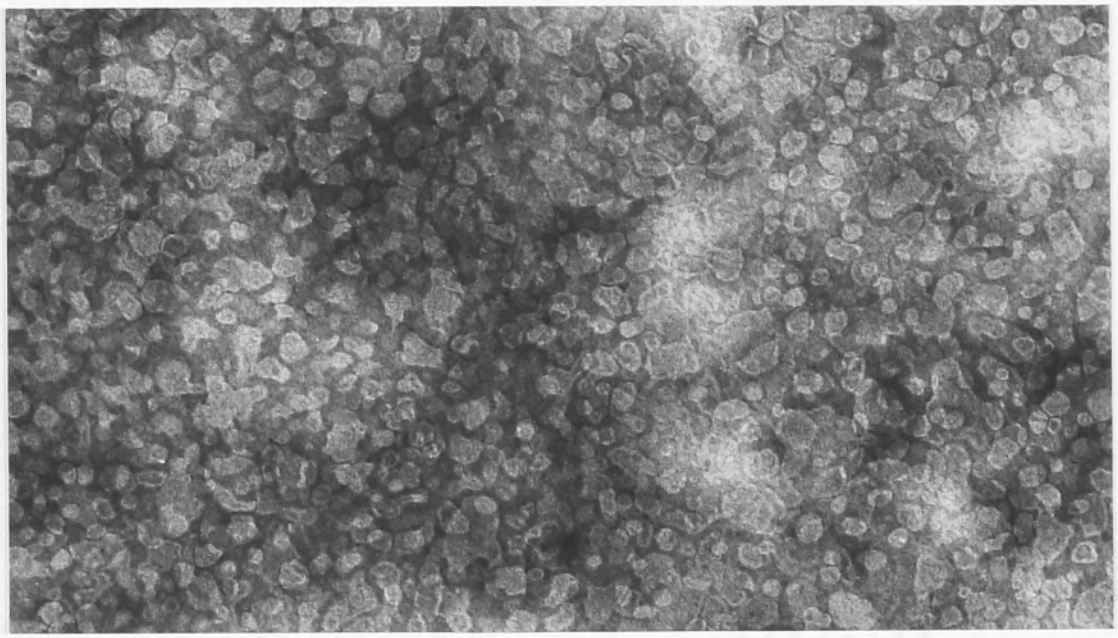
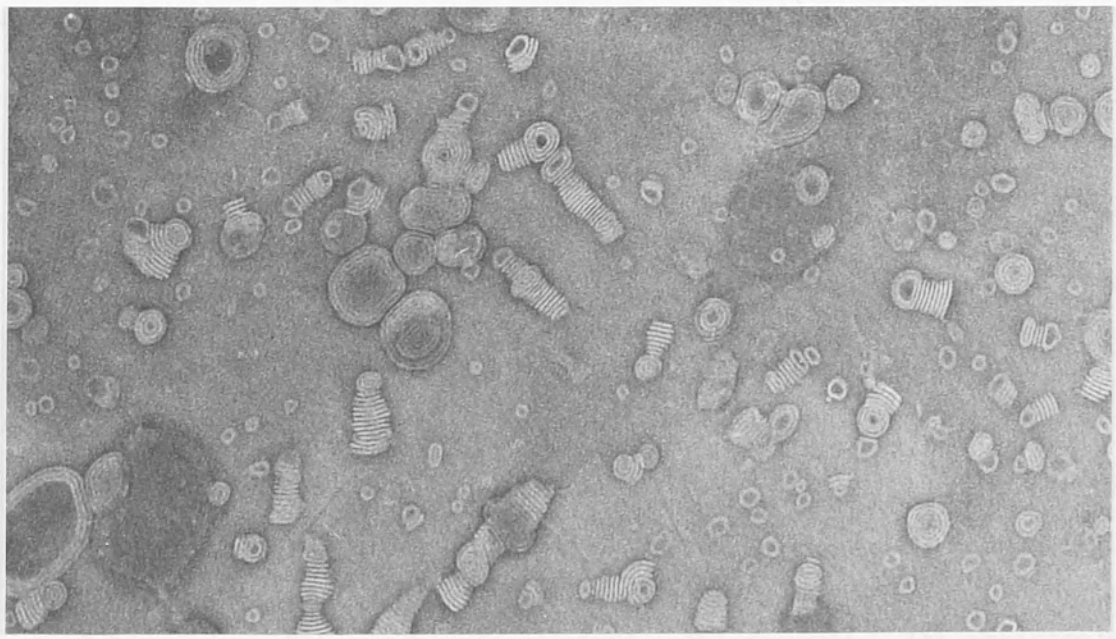


FIG. 3.9 (a) Negatively stained unilamellar liposomes of 9:1 phosphatidylcholine:phosphatidylserine, as viewed under the electron microscope.



(b) Partly sonicated lipid, showing some unilamellar liposomes, along with multilamellar structures.

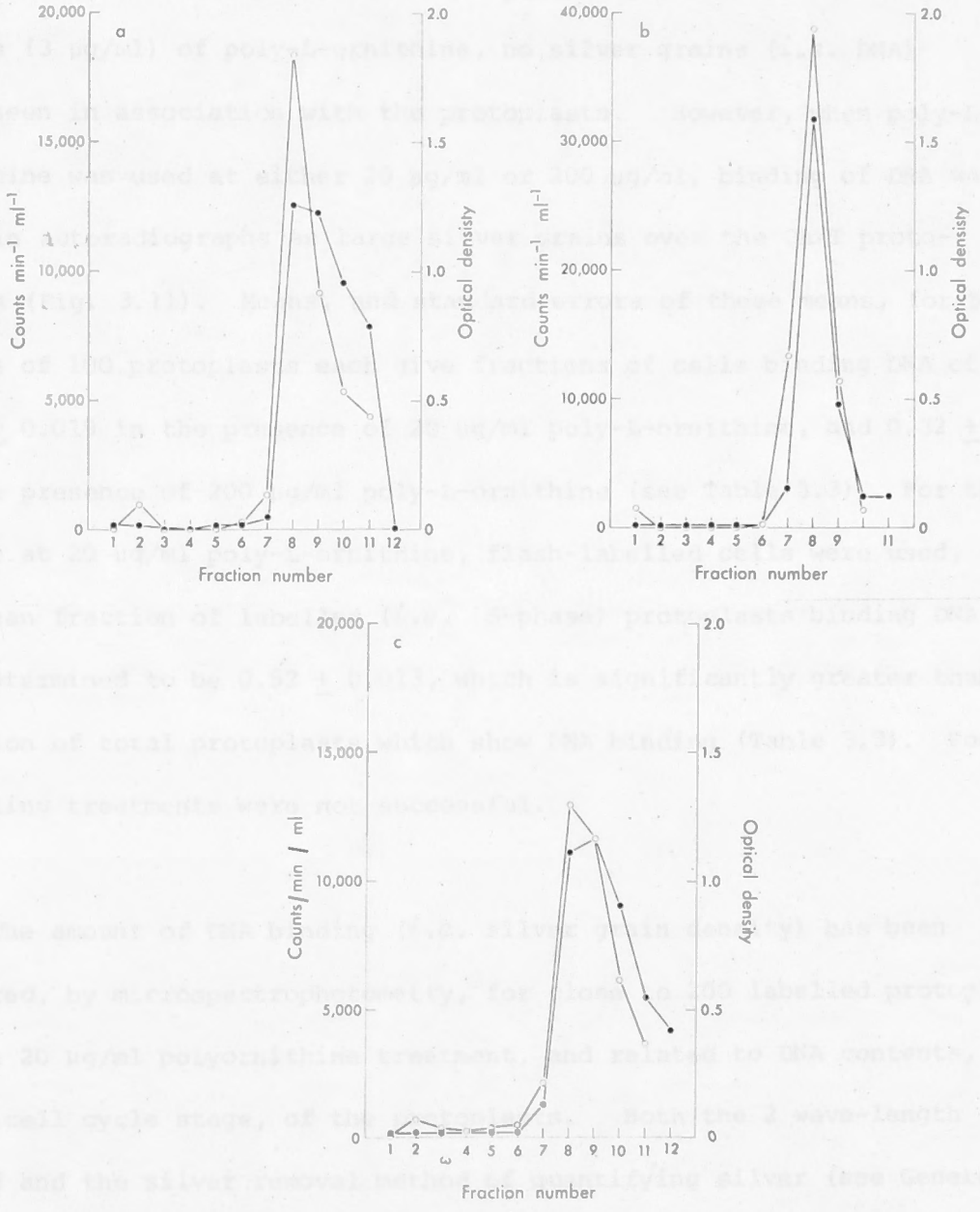


FIG. 3.10 Distribution of labelled cholesterol and lipid (given by optical density), along a step-wise ficoll gradient, in 3 samples of liposomes, (a) negatively charged, (b) neutral, (c) positively charged.

3.3.4 Binding of DNA and Liposomes to Protoplasts

DNA binding:-

Following treatment of CAPT protoplasts with DNA but no, or low levels (3 µg/ml) of poly-L-ornithine, no silver grains (*i.e.* DNA) were seen in association with the protoplasts. However, when poly-L-ornithine was used at either 20 µg/ml or 200 µg/ml, binding of DNA was seen in autoradiographs as large silver grains over the CAPT protoplasts (Fig. 3.11). Means, and standard errors of these means, for 5 counts of 100 protoplasts each give fractions of cells binding DNA of, 0.30 $\pm$ 0.019 in the presence of 20 µg/ml poly-L-ornithine, and 0.32 $\pm$ 0.019 in the presence of 200 µg/ml poly-L-ornithine (see Table 3.3). For the sample at 20 µg/ml poly-L-ornithine, flash-labelled cells were used, and the mean fraction of labelled (*i.e.* S-phase) protoplasts binding DNA was determined to be 0.52 $\pm$ 0.013, which is significantly greater than the fraction of total protoplasts which show DNA binding (Table 3.3). Post labelling treatments were not successful.

The amount of DNA binding (*i.e.* silver grain density) has been measured, by microspectrophotometry, for close to 200 labelled protoplasts of the 20 µg/ml polyornithine treatment, and related to DNA contents, and hence cell cycle stage, of the protoplasts. Both the 2 wave-length method and the silver removal method of quantifying silver (see General Materials and Methods) have been used, and the results have been plotted (Fig. 3.12). Results from the 2 different microspectrophotometry methods appear to be similar, with silver densities falling within the same range (0 → 7000) in both cases. Apparent differences in DNA contents (*i.e.* Feulgen density) may be attributed to differences in staining intensities

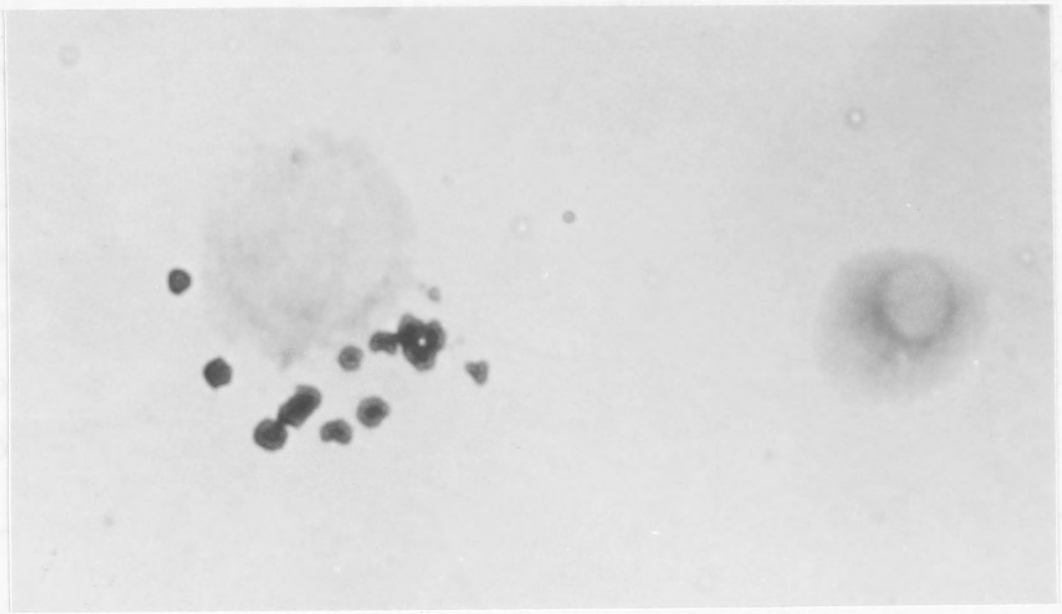


FIG. 3.11(a) A Feulgen stained autoradiograph of 2 CAPT protoplasts (only the nuclei are visible). The large silver grains attached to the left hand protoplast are visible indicators of DNA binding to this protoplast. Differences in nuclear state i.e. nuclear size and Feulgen density may be related to the differential binding observed.

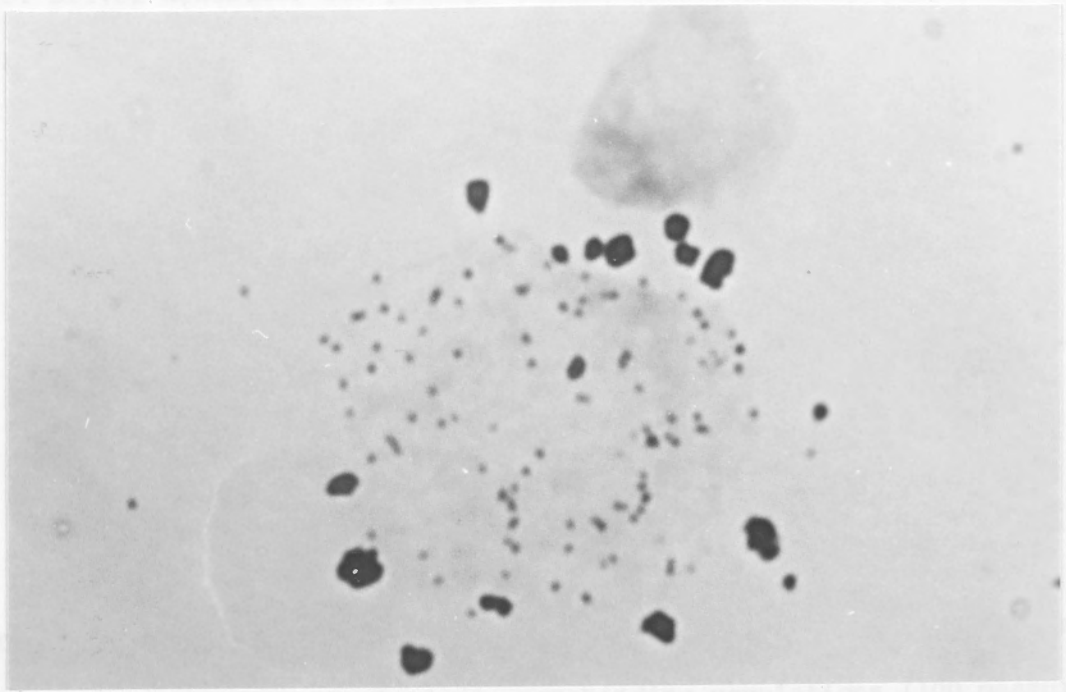


FIG. 3.11(b) DNA binding, in the form of large silver grains attached to a nuclear labelled, S-phase protoplast. No DNA is associated with the more contracted nucleus.

TABLE 3.3

Poly-L-ornithine (µg/ml)	Fraction of protoplasts showing binding (mean of 5 counts of 100 protoplasts each ± S.E.M.)	Fraction of prelabelled protoplasts (i.e. S-phase) showing binding (mean of 5 counts of 100 protoplasts each ± S.E.M.)
0	0	-
3	0	-
20	0.30 ± 0.019	0.52 ± 0.013
200	0.32 ± 0.019	-

of the 2 slides used. For both sets of data it can be seen that protoplasts of all 3 cycle stages, G_1, S, and G_2 (very few mitotic figures were seen) are represented in these labelled populations and hence all bind some DNA. Means of silver densities for protoplasts in each compartment of the cell cycle have been calculated (Table 3.4) and these indicate that:-

(i) G_2 protoplasts bind more DNA than G_1 protoplasts, as determined by both methods.

(ii) S-phase protoplasts show considerable variability in DNA binding, with high mean value by the 2 wavelength method and low mean value for the silver removal method. Such variability is probably due to low sample size rather than to any real phenomenon.

The fractions of protoplasts in each cell cycle compartment have been compared for the binding populations, non-binding populations (100 measurements each, taken from one of the microspectrophotometry slides) and the total population (binding plus non-binding) (see Table 3.4). These fractions were

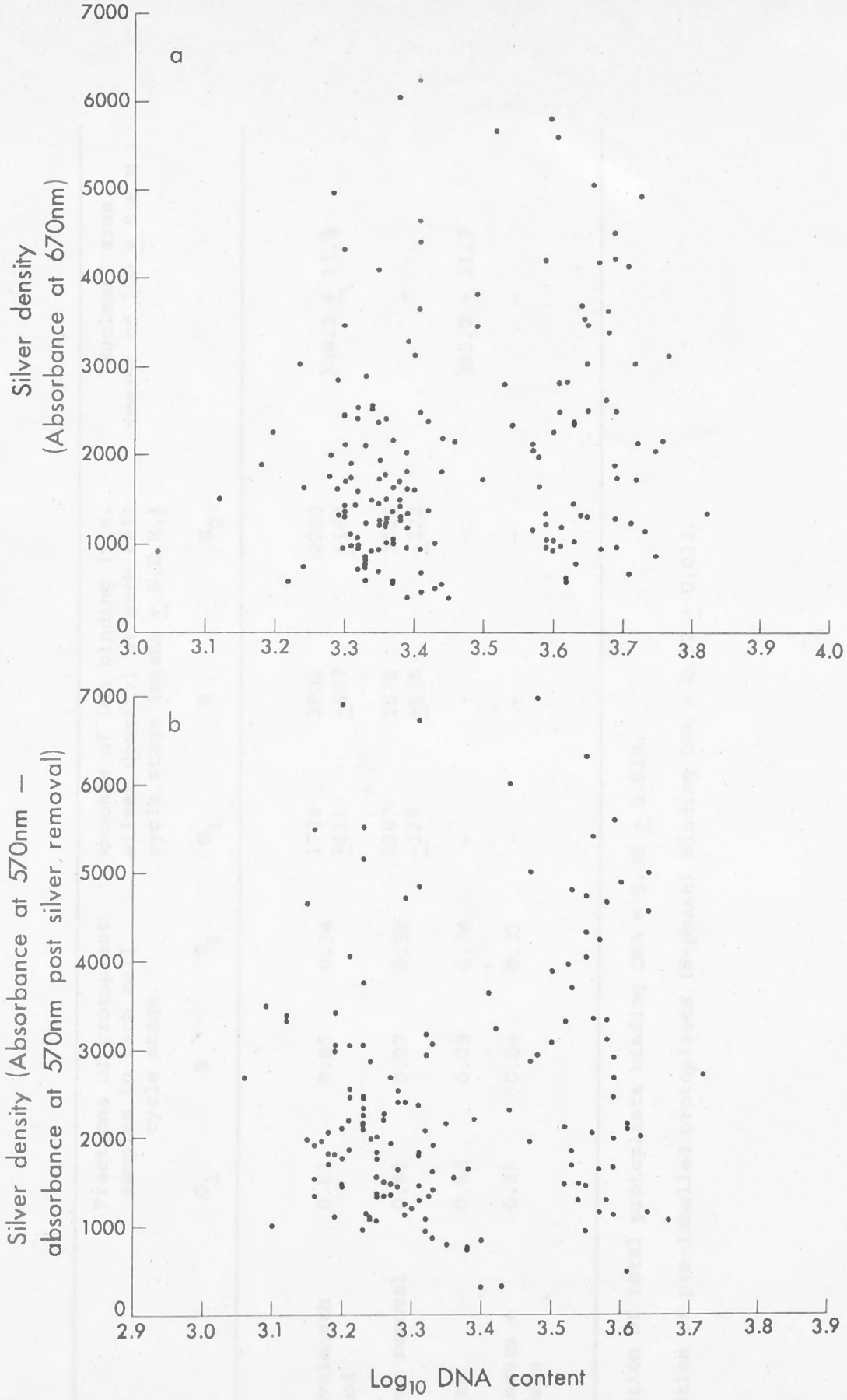


FIG. 3.12 Silver densities (*i.e.* amount of ^{3}H DNA binding) associated with 200 CAPT protoplasts of different cell cycle stages (as assessed by DNA content), measured by (a) 2-wavelength method, and (b) silver-removal method.

TABLE 3.4

Sample	Fractions of protoplast samples in each cell cycle stage			Amounts of DNA binding (*i.e.* silver density) at each cell cycle stage (means ± S.E.M.)			"Nuclear" area (means of 100 ± S.E.M.)
	G_1	S	G_2	G_1	S	G_2	
Binders							
(i) 2 wavelength method	0.61	0.05	0.34	1784 ±111	2802 ±557	2200 ±164	338.3 ± 11.3
(ii) silver removal	0.61	0.07	0.32	2287 ±128	2078 ±515	3025 ±222	-
Non-binders	0.63	0.03	0.34	-	-	-	393.3 ± 11.7
Total (binders + non-binders	0.65	0.04	0.31	-	-	-	-

Mean fraction of total protoplasts binding DNA = 0.30 ± 0.019.

Mean fraction of pre-labelled protoplasts (S-phase) binding DNA = 0.52 ± 0.013.

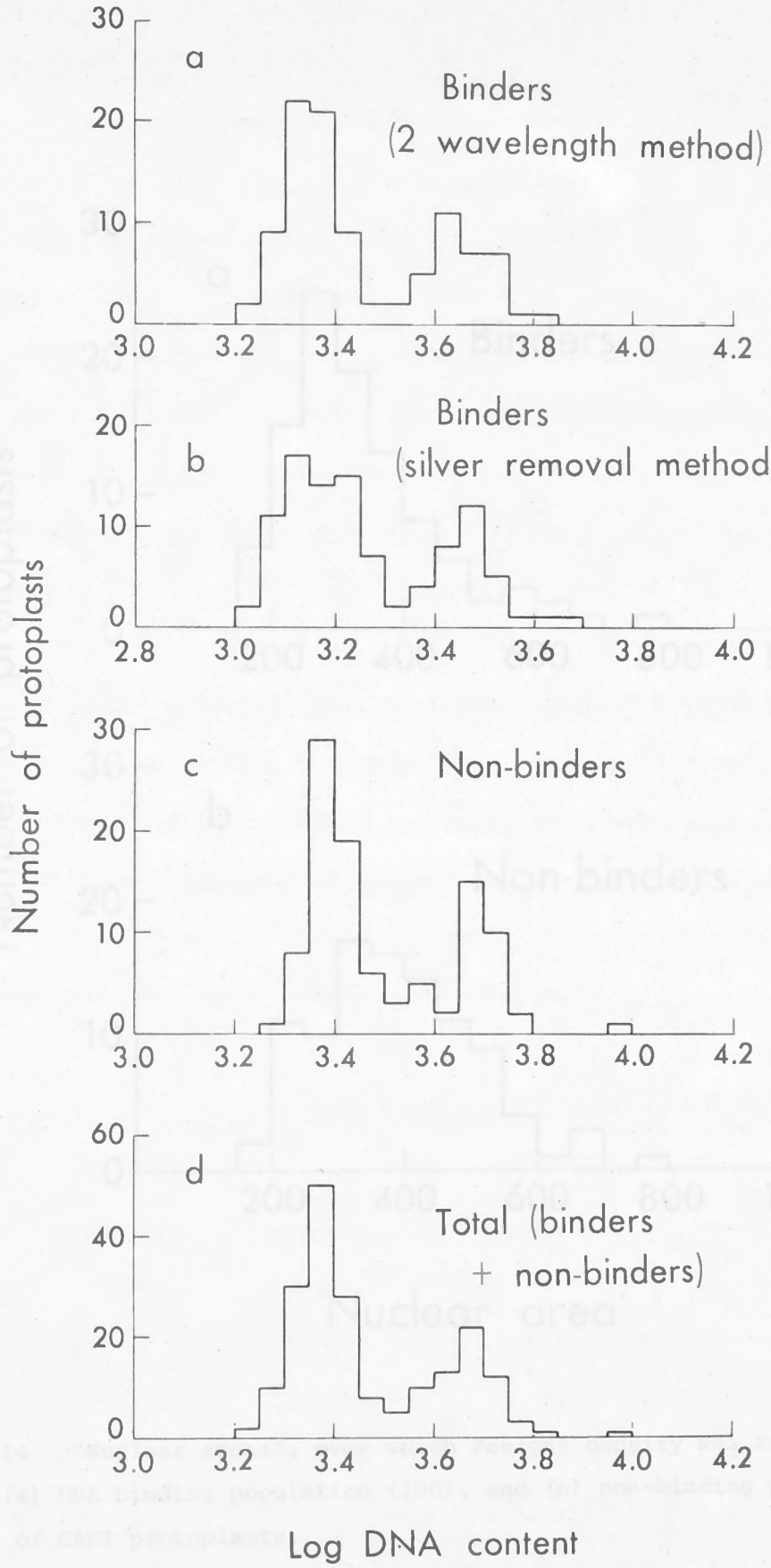


FIG. 3.13 DNA profiles of CAPT protoplasts trested with CAPT DNA and 20 μ g/L poly-L-ornithine. (a) Binding population (99), DNA contents determined by 2-wavelength method. (b) Binding population (100), DNA contents determined by silver removal method. (c) Non-binding population (101). (d) Total population (195).

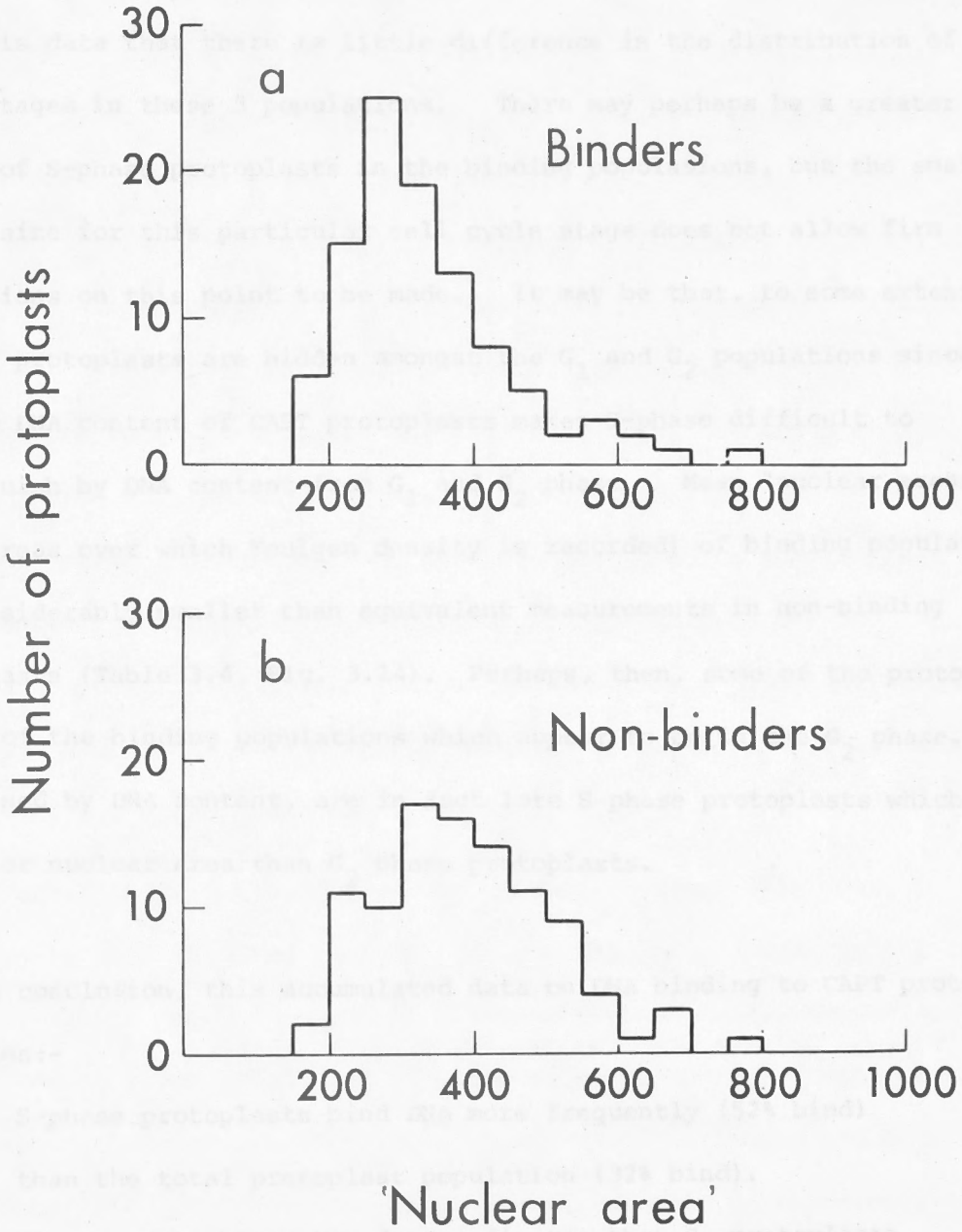


FIG. 3.14 "Nuclear areas", over which Feulgen density was recorded for (a) DNA binding population (100), and (b) non-binding population (99) of CAPT protoplasts.

obtained from microspectrophotometry data and also from DNA profiles for all 3 population types, which are presented in Fig. 3.13. It can be seen from this data that there is little difference in the distribution of cell cycle stages in these 3 populations. There may perhaps be a greater number of S-phase protoplasts in the binding populations, but the small sample size for this particular cell cycle stage does not allow firm conclusions on this point to be made. It may be that, to some extent, S-phase protoplasts are hidden amongst the G_1 and G_2 populations since the low DNA content of CAPT protoplasts makes S-phase difficult to distinguish by DNA content from G_1 and G_2 phase. Mean "nuclear areas" (*i.e.* areas over which Feulgen density is recorded) of binding populations are considerably smaller than equivalent measurements in non-binding protoplasts (Table 3.4, Fig. 3.14). Perhaps, then, some of the protoplasts of the binding populations which appear to be in the G_2 phase, as determined by DNA content, are in fact late S-phase protoplasts which have a smaller nuclear area than G_2 phase protoplasts.

In conclusion, this accumulated data on DNA binding to CAPT protoplasts indicates:-

(i) S-phase protoplasts bind DNA more frequently (52% bind) than the total protoplast population (32% bind).

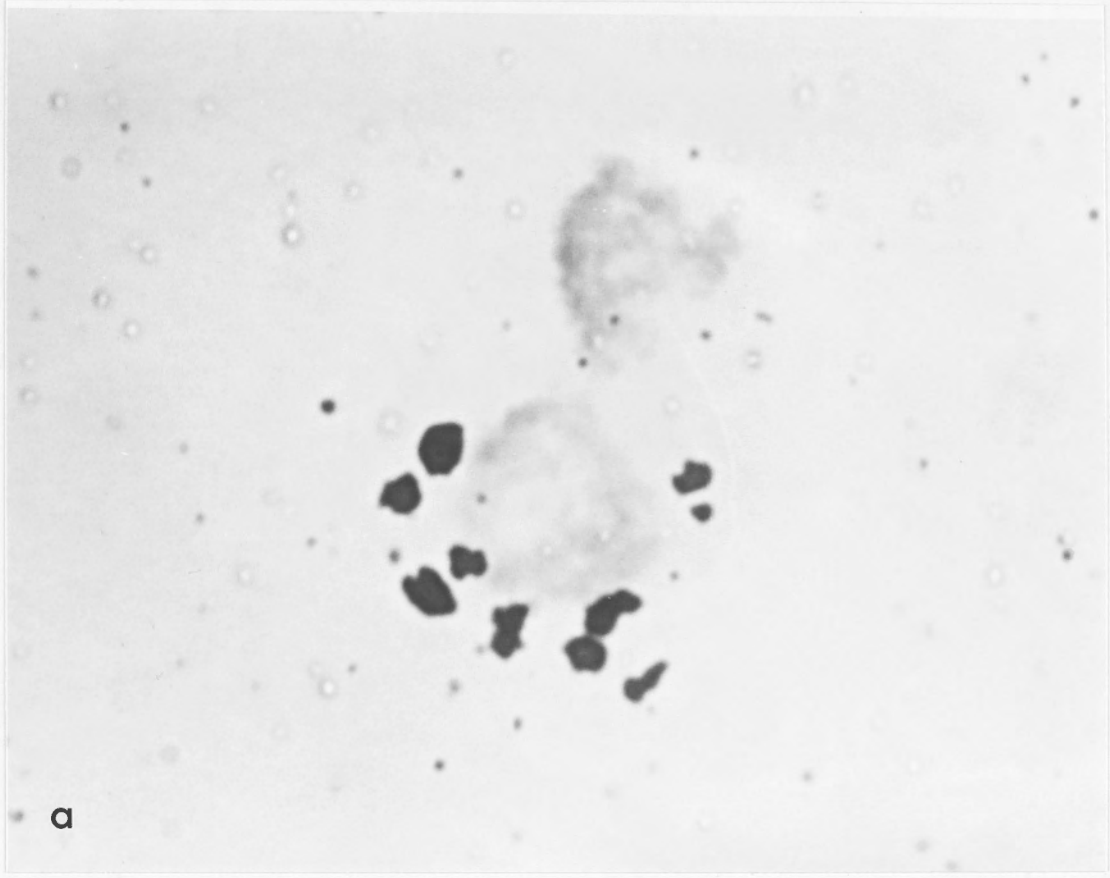
(ii) Microspectrophotometry data indicates that G_2 protoplasts generally bind more DNA than G_1 protoplasts, although less than might be expected based on surface area differences.

(iii) The fractions of G_1 and G_2 phase protoplasts in the binding population do not deviate considerably from normal *i.e.* total population.

Liposome binding:-

Binding of liposomes to both CAPT and NS-1 protoplasts was seen as large silver grains attached to protoplasts in autoradiograph preparations (see Fig. 3.15). For all 3 liposome types used, no binding was achieved in the absence of poly-L-ornithine or at low levels (3 µg/ml) of poly-L-ornithine. Between 30% and 40% of CAPT protoplasts showed binding for all 3 types of liposomes in the presence of 20 µg/ml poly-L-ornithine (see Table 3.5). Similarly, 32% of NS-1 protoplasts had bound negatively charged liposomes. A considerably higher fraction of CAPT protoplasts showed binding with positively charged and neutral liposomes than with negatively charged liposomes, and slightly less binding was achieved with protoplasts from flash labelled cells (*i.e.* S-phase protoplasts) than for the total population with both neutral and positively charged liposomes (see Table 3.5). All measurements recorded in Table 3.5 are means of 5 counts of 100 protoplasts each.

Further analysis was made on the binding of negatively charged liposomes to both CAPT and NS-1, in the presence of 20 µg/ml poly-L-ornithine. Using the silver removal method of microspectrophotometry, comparison of silver density (*i.e.* amounts of liposome binding) and DNA contents, and hence cell cycle stage, of binding protoplasts of both CAPT and NS-1 (100 protoplasts each) has been made. Plots of this data are presented in Fig. 3.16. As with DNA binding, this data indicates that all 3 cell cycle stages, G_1, S, and G_2 (very few mitotic figures were seen) are represented in the binding populations of both CAPT and NS-1. Mean silver densities (*i.e.* amounts of liposome binding) for protoplast samples in each cell cycle compartment have been calculated for both populations (see Table 3.6), and these measurements indicate:-



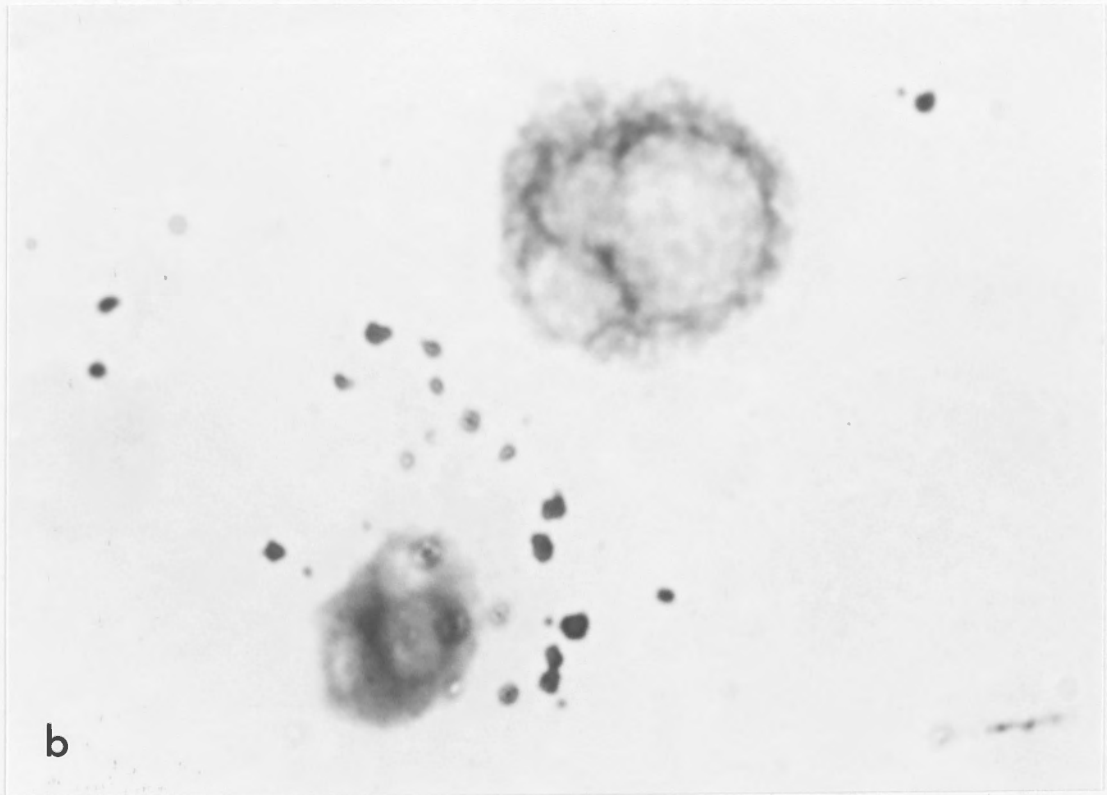


FIG. 3.15. Differential liposome binding, as indicated by large silver grains, with Feulgen stained protoplasts of (a) CAPT, and (b) NS-1.

TABLE 3.5

	Fraction of total protoplasts showing binding (mean of 5 counts of 100 protoplasts each ± S.E.M.)	Fraction of pre-labelled protoplasts (*i.e.* S-phase) showing binding (mean of 5 counts of 100 protoplasts each ± S.E.M.)
CAPT (+0.1 ml liposomes)		
Liposome Type		
-vely charged	0.346 ± 0.0035	-
neutral	0.454 ± 0.032	0.418 ± 0.03
+vely charged	0.448 ± 0.035	0.384 ± 0.0098
CAPT (+0.1 ml liposomes)		
Liposome Type		
-vely charged	0.384 ± 0.015	-
NS-1 (+0.5 ml liposomes)		
Liposome Type		
-vely charged	0.326 ± 0.042	-

(i) NS-1 protoplasts consistently bind greater quantities (approximately double) of the negatively charged liposomes as compared with the CAPT protoplasts.

(ii) G_2 protoplasts bind more liposomes than G_1 protoplasts in both CAPT and NS-1 populations.

(iii) S-phase protoplasts bind at levels closer to G_1 than to G_2.

The fractions of protoplasts in each cell cycle compartment have been compared for binding populations, non-binding protoplasts (100 measurements each from microspectrophotometry slides), and the total population (binding plus non-binding) (Table 3.6). These fractions were obtained from microspectrophotometry data and also from DNA profiles of all 3 population types, which are presented in Fig. 3.17. This data indicates that in both CAPT and NS-1, binding populations have a greater number of G_2's and fewer G_1's than the other populations. There may also be fewer S-phase protoplasts in the binding population of NS-1. Unfortunately the cells used in binding negatively charged liposomes were not pre-labelled, so that no data is available on the frequency of binding in an S-phase population as compared with the total population.

There are no significant differences in "nuclear areas" between binding and non-binding populations of NS-1 or CAPT (Fig. 3.18).

In summary liposome binding data indicates:-

(i) All 3 liposome types require poly-L-ornithine for binding to protoplasts.

(ii) S-phase protoplasts of CAPT bind positively charged and neutral liposomes less frequently than the total population.

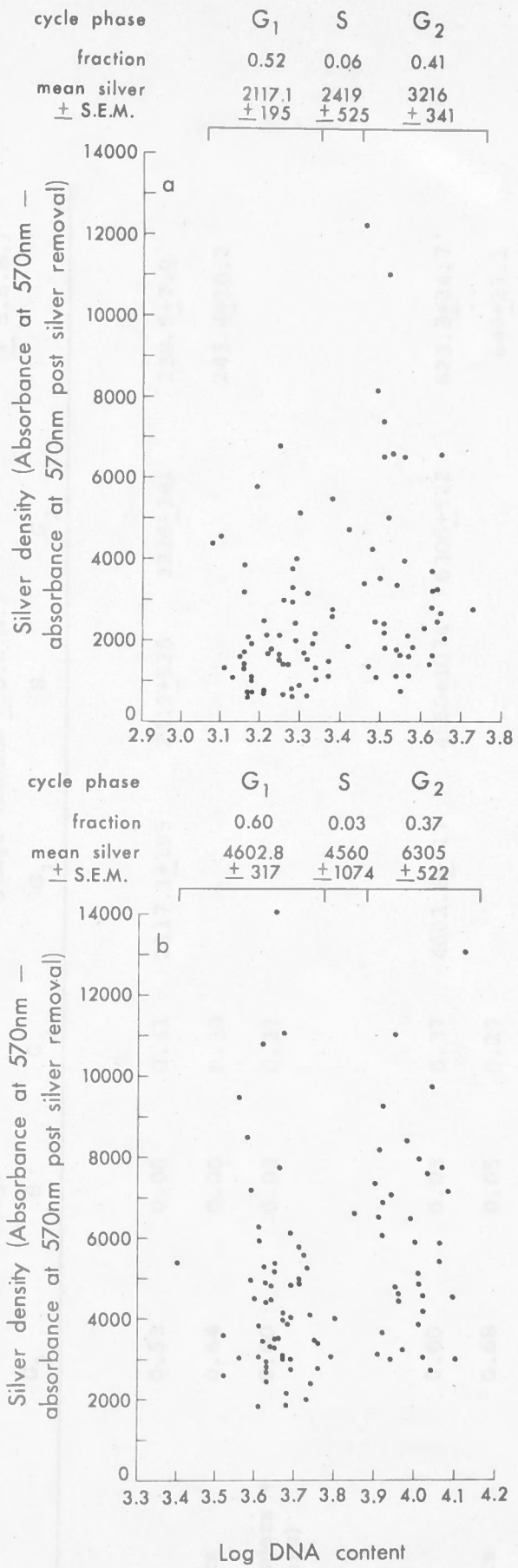


FIG. 3.16 The binding of labelled negatively charged liposomes, in the presence of 20 μg/L poly-L-ornithine, (*i.e.* silver density), to (a) 100 CAPT protoplasts, and (b) 100 NS-1 protoplasts of different cell cycle stages (as assessed by DNA content). Silver densities were determined using the silver removal method.

TABLE 3.6 - ANALYSIS OF LIPOSOME BINDING

Sample	Fractions of protoplast samples in each cell cycle stage			Amounts of liposome binding (*i.e.* silver density) at each cell cycle stage (means ± S.E.M.)			"Nuclear" area (means of 100 ± S.E.M.)
	G_1	S	G_2	G_1	S	G_2	
CAPT:							
Binders	0.53	0.06	0.41	2117.1±195	2419±525	3216±341	238.5±7.9
Non-binders	0.64	0.06	0.33				243.4±10.2
Total (binders + non-binders)	0.60	0.03	0.37				
NS-1:							
Binders	0.60	0.03	0.37	4602.8±317	4560±1074	6305±522	623.3±24.7
Non-binders	0.68	0.05	0.27				649±21.1
Total (binders + non-binders)	0.62	0.08	0.30				

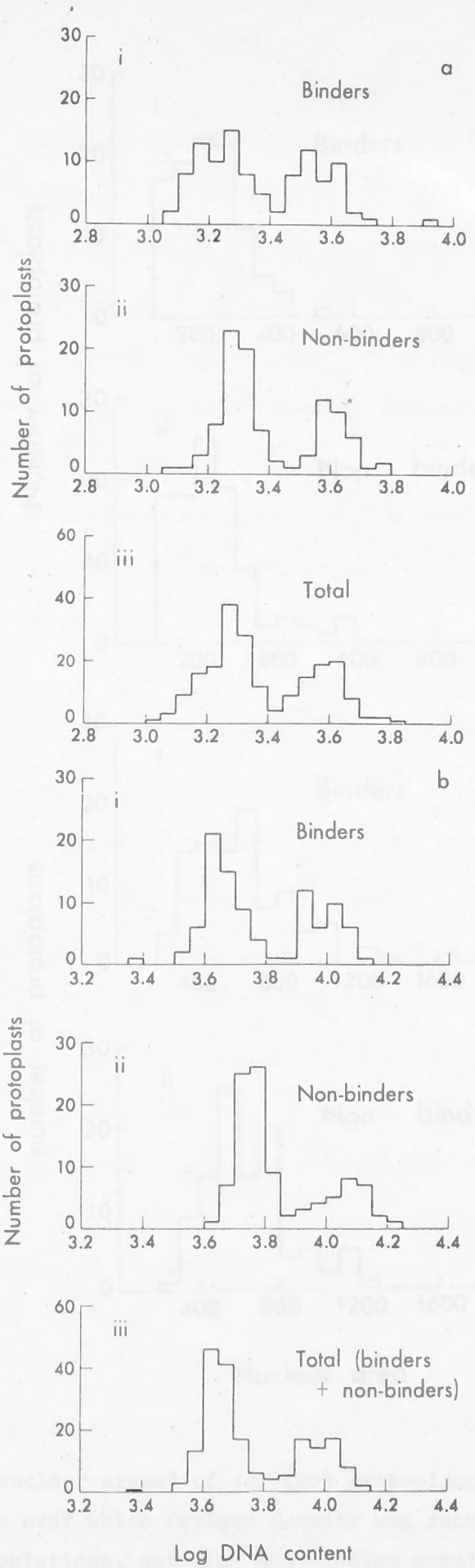


FIG. 3.17 DNA profiles of (a) CAPT protoplasts, and (b) NS-1 protoplasts treated with negatively charged liposomes in the presence of 20 μg/L poly-L-ornithine. (i) Binding populations, (ii) non-binding populations, and (iii) total populations.

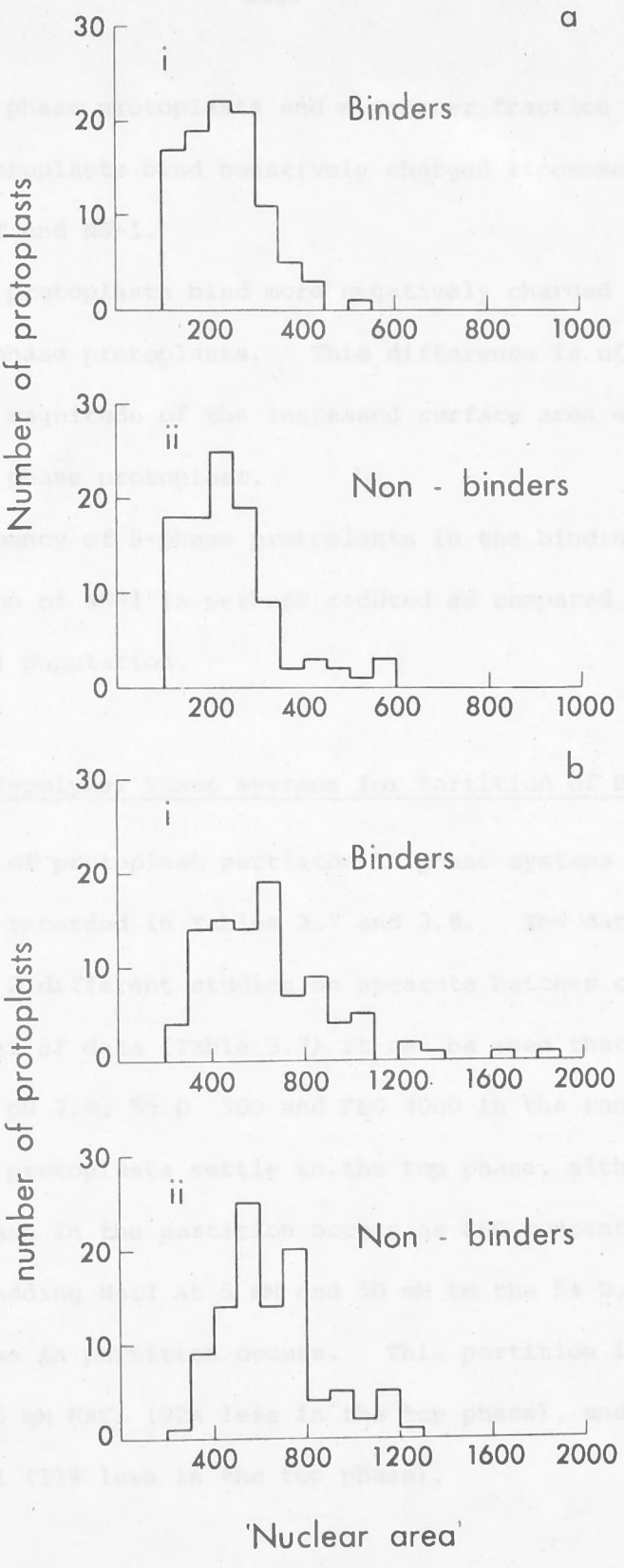


FIG. 3.18 "Nuclear areas" of (a) CAPT protoplasts, and (b) NS-1 protoplasts over which Feulgen density was recorded for (i) liposome binding populations, and (ii) non-binding populations.

(iii) Fewer G_1 phase protoplasts and a greater fraction of G_2 phase protoplasts bind negatively charged liposomes for both CAPT and NS-1.

(iv) G_2 phase protoplasts bind more negatively charged liposomes than G_1 phase protoplasts. This difference is of the order of magnitude of the increased surface area expected for a G_2 phase protoplast.

(v) The frequency of S-phase protoplasts in the binding population of NS-1 is perhaps reduced as compared with the total population.

3.3.5 Aqueous 2-polymer Phase systems for Partition of Protoplasts

The results of protoplast partition in phase systems of various compositions are recorded in Tables 3.7 and 3.8. The data in each table represents 2 different studies on spearate batches of protoplasts. From the first set of data (Table 3.7) it can be seen that, using a 10 mMkPO_4 buffer pH 7.8, 5% D 500 and PEG 4000 in the range 5% to 9% close to 100% of protoplasts settle in the top phase, although a noticeable decrease in the partition occurs as PEG concentration is increased. By adding NaCl at 5 mM and 50 mM to the 5% D, 7% PEG phase system, a decrease in partition occurs. This partition is greatly decreased with 50 mM NaCl (92% less in the top phase), and much less so with 5 mM NaCl (11% less in the top phase).

The effect of temperature on partition was also tested, and the results are recorded in Table 3.7 and Fig. 3.19. At room temperature increased partition is obtained as compared with that obtained at 4°C.

TABLE 3.7 - RESULTS OF SINGLE TUBE 2-POLYMER PHASE SEPARATION OF NS-1 PROTOPLASTS

% Dextran 500 / % PEG 4000		A^T	A^B	Top volume	Bottom volume	Bottom sample volume	$X(A^T \times 3)$	$Y(A^B \times$ [bottom sample volume + 2.5 ml])	$\Sigma(X+Y)$	Amount in top ($A^T \times$ dil.factor $\times$ top phase volume)	% in top
5/5		0.516	0.083	1.8	2.0	2.3	1.548	0.398	1.946	1.858	95.5
5/7		0.524	0.085	1.8	2.0	2.3	1.572	0.408	1.980	1.886	95.3
5/9		0.469	0.112	1.8	2.0	2.3	1.407	0.538	1.945	1.688	86.8
	NaCl(mM)										
5/7	0	0.524	0.085	1.8	2.0	2.3	1.572	0.408	1.980	1.886	95.3
5/7	5	0.427	0.132	1.9	1.9	2.3	1.281	0.634	1.915	1.623	84.7
5/7	50	0.021	0.363	1.8	2.0	2.3	0.633	1.742	2.375	0.076	3.2
% Dextran 500 / % PEG 6000											
	Temp.										
5/3.5	(∿20°C) room	0.369	0.277	2.4	1.4	2.3	1.107	1.329	2.436	1.771	72.7
5/3.5	4°C	0.290	0.205	2.0	1.8	2.3	0.87	0.984	1.854	1.16	62.3
5/4	room	0.05	0.361	2.0	1.8	2.3	0.15	0.733	1.883	0.20	10.6
5/4	4°C	0.005	0.364	2.15	1.65	2.3	0.015	1.747	1.762	0.024	1.36

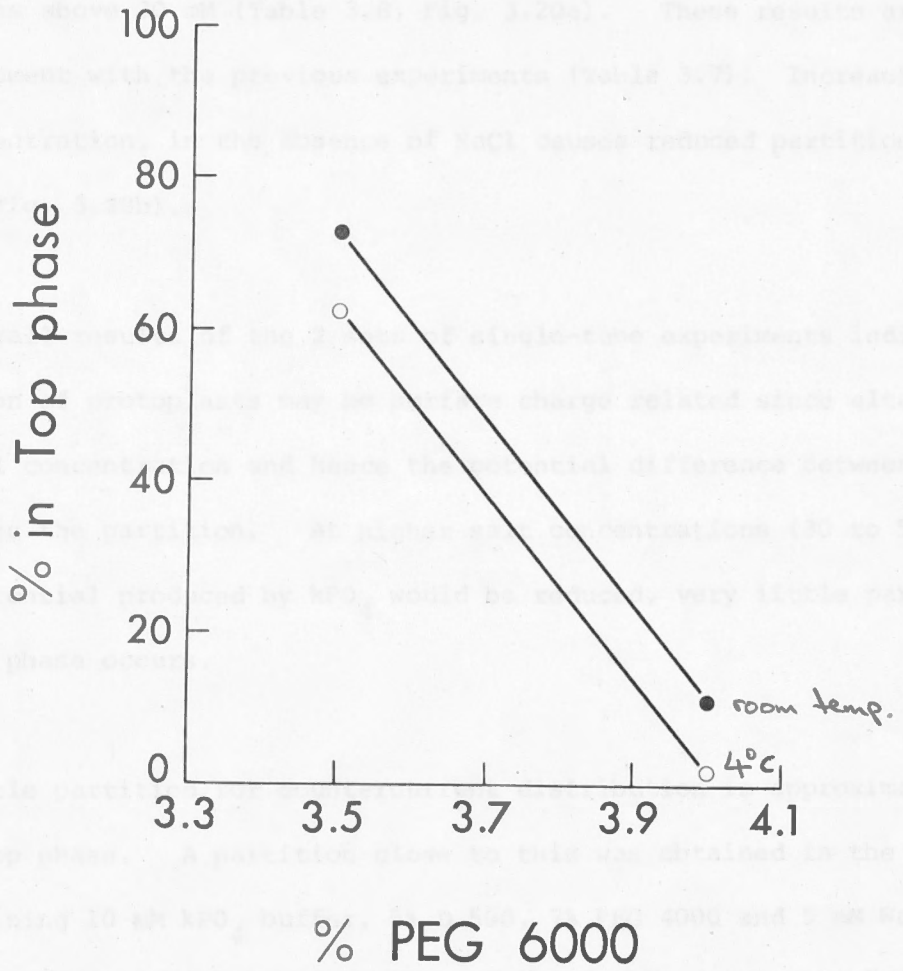


FIG. 3.19 Partition of NS-1 protoplasts in 2 different phase systems, at room temperature and 4°C.

In the second series of single-tube experiments the effect of varying NaCl concentrations was more thoroughly investigated on a 5% D 500, 3% PEG 6000 phase system. Results show that the addition of NaCl reduced the percentage of protoplasts in the top phase, down to zero at NaCl concentrations above 30 mM (Table 3.8, Fig. 3.20a). These results are thus in agreement with the previous experiments (Table 3.7). Increasing the PEG concentration, in the absence of NaCl causes reduced partition (Table 3.8, Fig. 3.20b).

The overall results of the 2 sets of single-tube experiments indicate that partition of protoplasts may be surface charge related since alteration of NaCl concentration and hence the potential difference between phases affects the partition. At higher salt concentrations (30 to 50 mM), where the potential produced by kPO_4 would be reduced, very little partition into the top phase occurs.

A suitable partition for countercurrent distribution is approximately 80% in the top phase. A partition close to this was obtained in the phase system containing 10 mM kPO_4 buffer, 5% D 500, 7% PEG 4000 and 5 mM NaCl (Table 3.7). Samples of top and bottom phases of this sytem were Feulgen stained, and DNA content distributions for these samples are presented in Fig. 3.21. 50 measurements were made on each fraction. Profiles for top and bottom phases are similar, although there appear to be more protoplasts having the G_1 DNA content in the lower phase, and there is a gap in the DNA distribution of this profile, which indicates that there are fewer S-phase cells in this fraction. However, the populations of protoplasts in the 2 phases do not display a distinct separation into populations at different cell cycle stages.

TABLE 3.8 - RESULTS OF SINGLE TUBE 2-POLYMER PHASE SEPARATIONS OF NS-1 PROTOPLASTS

% Dextran 500 / % PEG 6000	NaCl (mM)	A^T	A^B	Top volume (used mean)	Bottom volume (mean value)	Bottom sample volume (B.S.V.)	$X(A^T \times 3)$	$Y(A^B \times [B.S.V.+2.5]$	$\Sigma(X+Y)$	Amount in top	% in top
5 / 3.0	0	0.775	0.049	1.87	1.83	2.2 [(1.87+1.84) - 1.5]	2.325	0.230	2.56	2.90	113
5 / 3.0	5	0.732	0.099	"	"	"	2.196	0.465	2.66	2.738	103
5 / 3.0	10	0.507	0.230	"	"	"	1.521	1.081	2.602	1.90	72.9
5 / 3.0	20	0.113	0.417	"	"	"	0.339	1.960	2.299	0.423	21.6
5 / 3.0	30	0	0.526	-	-	-	-	-	-	-	0
5 / 3.0	40	0	0.513	-	-	-	-	-	-	-	0
5 / 3.0	50	0.009	0.481	1.87	1.83	2.2 [(1.87+1.84) - 1.5]	0.027	2.261	2.288	0.0337	1.5
5 / 3.55	0	0.312	0.190	1.28	2.43	2.21	0.936	0.895	1.831	0.799	43.6
5 / 3.70	0	0.246	0.180	"	"	"	0.738	0.848	1.586	0.630	39.7
5 / 3.85	0	0.192	0.217	"	"	"	0.576	1.022	1.598	0.492	30.8
5 / 4.0	0	1.119	0.228	"	"	"	0.357	1.074	1.431	0.305	21.3

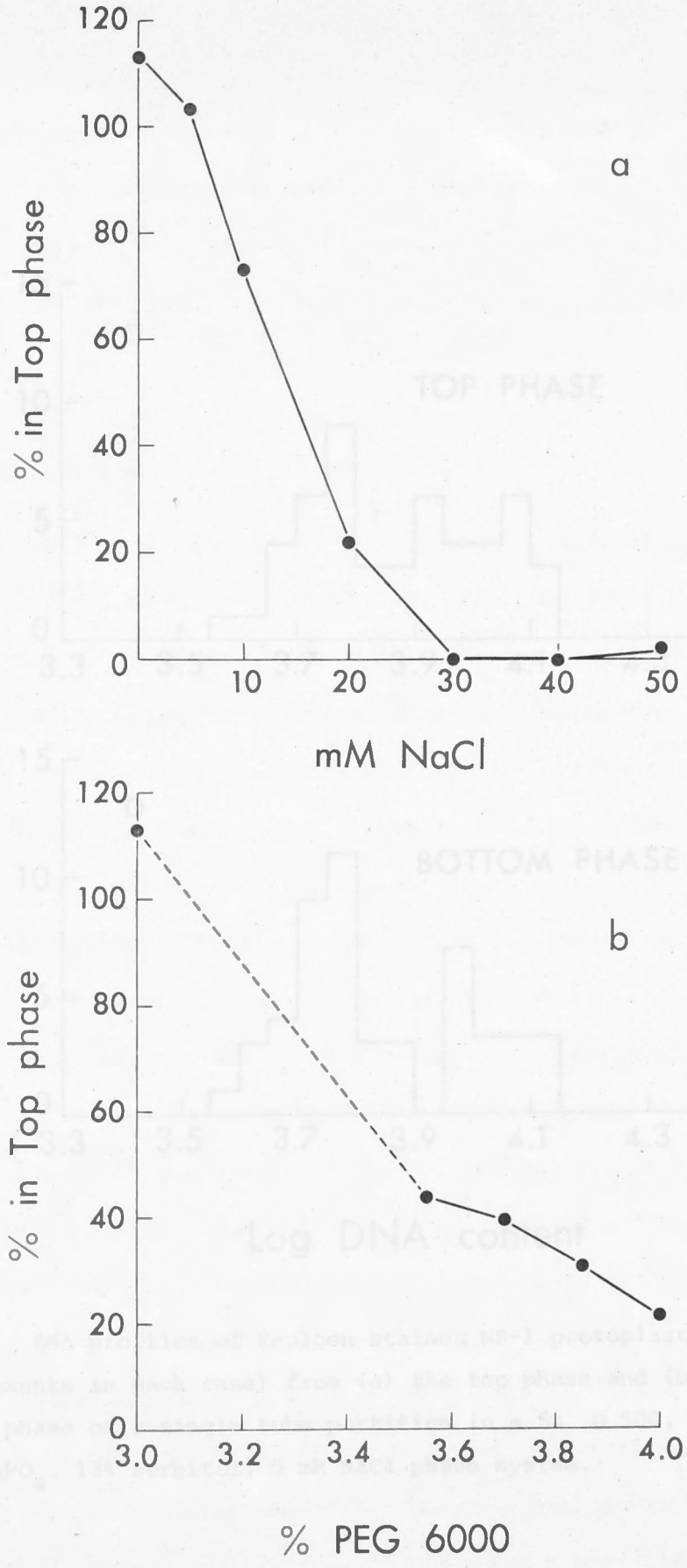


FIG. 3.20 (a) Percentage of NS-1 protoplasts in the top phase of a 5% D500, 3% PEG 6000, 10 mM kPO_4, 13% sorbitol, system, with varying NaCl concentrations.

(b) Percentage of NS-1 protoplasts in the top phase of a system containing 5% D500, 10 mM kPO_4, 13% sorbitol and varying

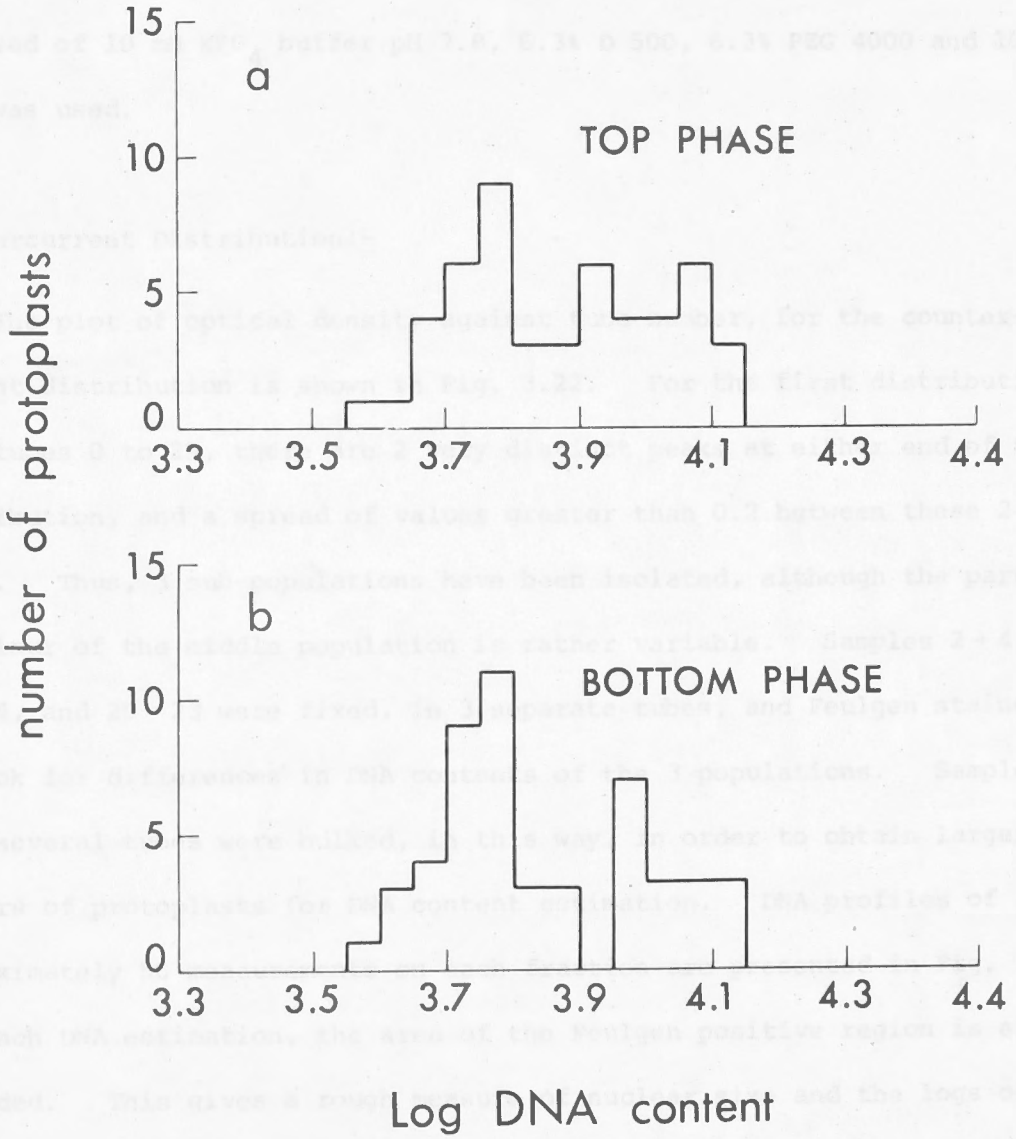


FIG. 3.21 DNA profiles of Feulgen stained NS-1 protoplasts (50 measurements in each case) from (a) the top phase and (b) the bottom phase of a single tube partition in a 5% D 500, 7% PEG 4000, 10 mM kPO_4, 13% sorbitol, 5 mM NaCl phase system.

The single-tube experiments thus give an indication that there may be a cell cycle related partition, but a countercurrent distribution is required to give a more effective separation. A phase system composed of 10 mM kPO_4 buffer pH 7.8, 6.3% D 500, 6.3% PEG 4000 and 10 mM NaCl was used.

Countercurrent Distribution:-

The plot of optical density against tube number, for the countercurrent distribution is shown in Fig. 3.22. For the first distribution *i.e.* tubes 0 to 29, there are 2 very distinct peaks at either end of the distribution, and a spread of values greater than 0.2 between these 2 peaks. Thus, 3 sub-populations have been isolated, although the partition behaviour of the middle population is rather variable. Samples 2→4, 12→14, and 20→23 were fixed, in 3 separate tubes, and Feulgen stained to look for differences in DNA contents of the 3 populations. Samples from several tubes were bulked, in this way, in order to obtain larger numbers of protoplasts for DNA content estimation. DNA profiles of approximately 50 measurements on each fraction are presented in Fig. 3.23. For each DNA estimation, the area of the Feulgen positive region is also recorded. This gives a rough measure of nuclear size and the logs of these values are plotted as histograms for each sub-population in Fig. 3.24.

It can be seen from Fig. 3.23 that the 3 populations are distinguishable in terms of their DNA content profiles. Whilst the profiles from fractions 2→4 and 12→14, are biphasic indicating the presence of protoplasts in both the G_1 and the G_2 state, the fraction 20→23 shows a monophasic DNA content profile. Since the DNA contents of the sub-population in tubes 20→23 are not only clustered into 1 peak but also have DNA contents intermediate

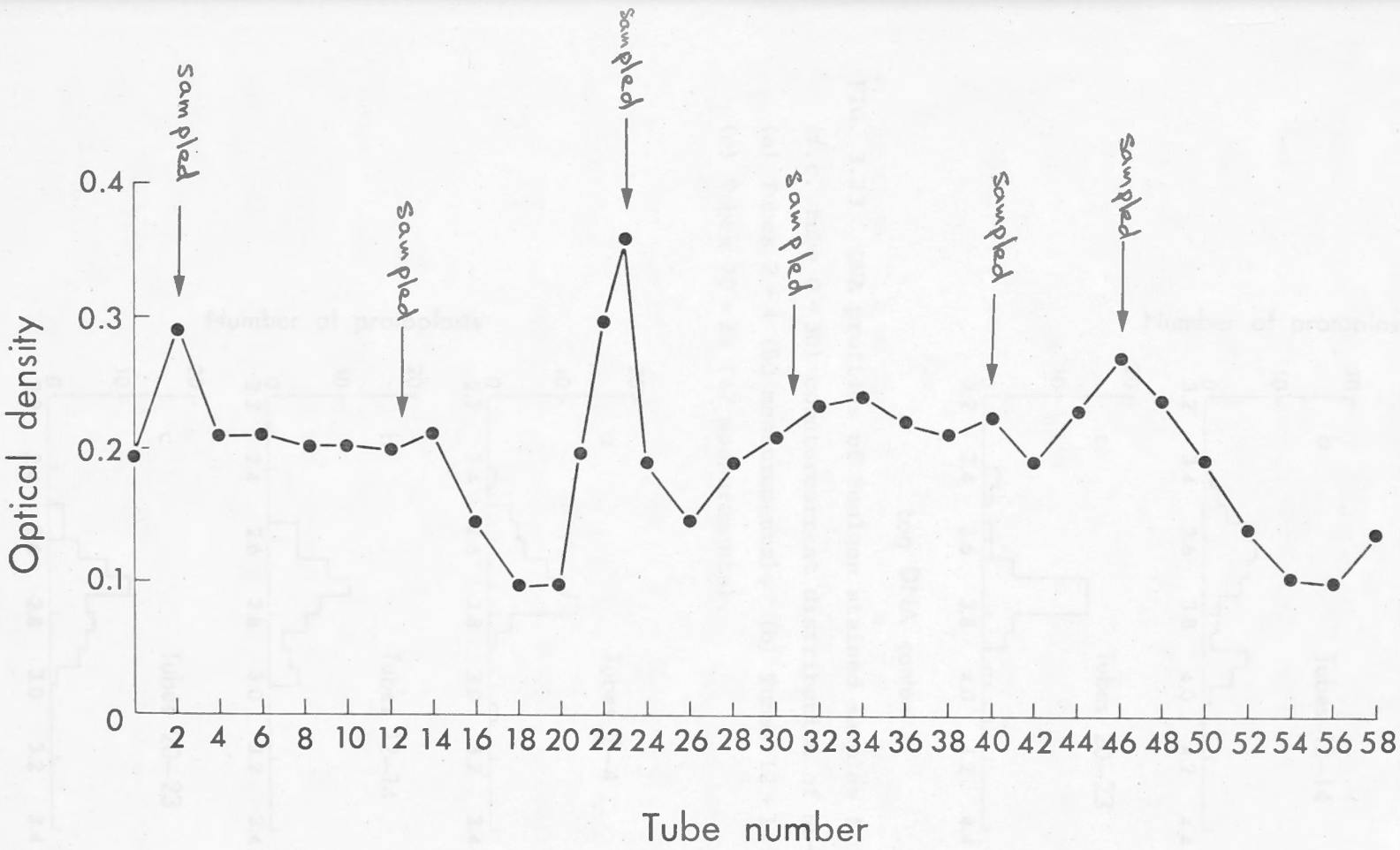


FIG. 3.22 Optical densities (*i.e.* protoplast density) over 2 × 30 tube countercurrent distributions of NS-1 protoplasts. The phase system is composed of 6.3% D500, 6.3% PEG 6000, 10 mM kPO_4, 13% sorbitol and 10 mM NaCl.

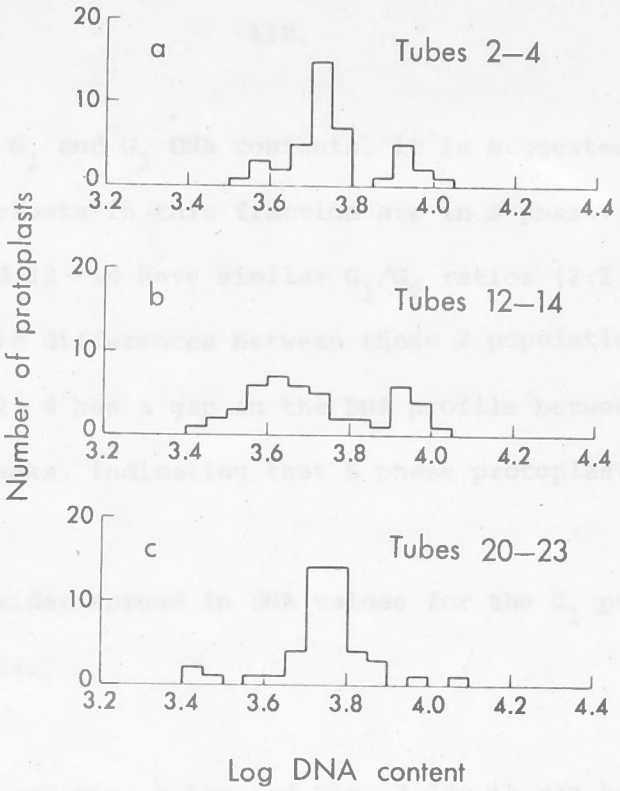


FIG. 3.23 DNA profiles of Feulgen stained samples from the first (*i.e.* tube 0 → 30) countercurrent distribution of NS-1 protoplasts. (a) Tubes 2 → 4 (50 measurements), (b) Tubes 12 → 14 (47 measurements), (c) Tubes 20 → 23 (47 measurements).

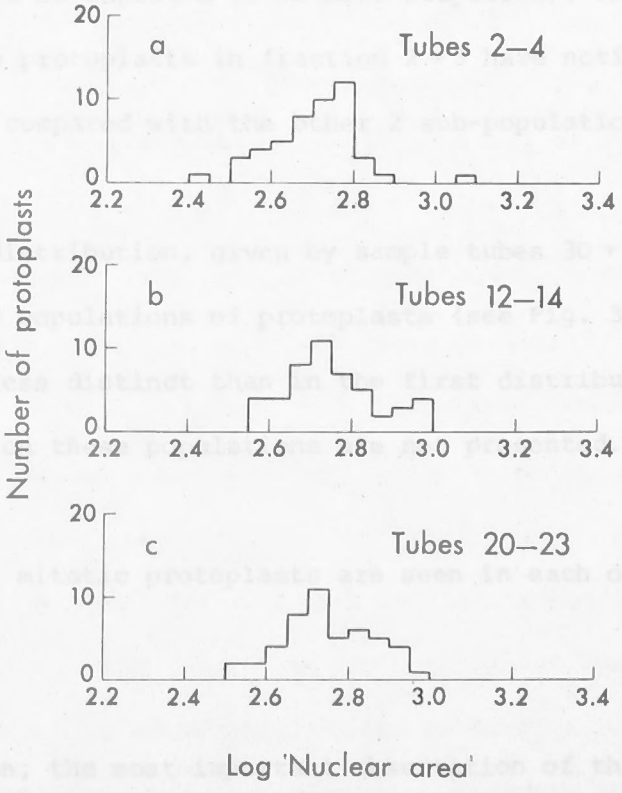


FIG. 3.24 "Nuclear areas" over which Feulgen density was recorded for samples from the countercurrent distribution of NS-1 protoplasts. (a) Tubes 2 → 4 (48 measurements), (b) Tubes 12 → 14 (48 measurements), and (c) Tubes 20 → 23 (48 measurements).

in values between G_1 and G_2 DNA contents, it is suggested that the majority of protoplasts in this fraction are in S-phase. Both of fractions 2→4 and 12→14 have similar G_1/G_2 ratios (2.1, 2.4 respectively). The most noticeable differences between these 2 populations are:-

(1) Population 2→4 has a gap in the DNA profile between the G_1 and G_2 peaks, indicating that S phase protoplasts may be absent.

(2) There is a wider spread in DNA values for the G_1 peak in sample 12→14.

By comparison of Fig. 3.23c and Fig. 3.24c it can be seen that "nuclear areas" of many protoplasts in the fraction 20→23 are much larger than protoplasts of similar DNA contents in other fractions. This gives further support to the apparent enrichment for S-phase protoplasts (in which nuclei would be expected to be more dispersed), in tubes 20→23. Additionally, the protoplasts in fraction 2→3 have noticeably smaller Feuglen area, as compared with the other 2 sub-populations.

The second distribution, given by sample tubes 30→59, also shows separation into 3 populations of protoplasts (see Fig. 3.22). However, these peaks are less distinct than in the first distribution, so that further analyses on these populations are not presented.

Less than 1% mitotic protoplasts are seen in each of the 6 sub-populations.

In conclusion, the most important observation of this work is that S-phase cells appear to partition differentially, and, in particular,

they tend to remain in the top phase, being transferred to the end cavities in the countercurrent distribution. There was also an indication, in a single tube experiment that S-phase cells were preferentially partitioned into the top phase (see Fig. 3.21). Differential surface charge of S-phase cells, as compared with G_1 and G_2 phase cells, may be an important factor in such partition, since a charged phase system was used. However, differences in hydrophobicity cannot be excluded, although since membranes usually have a high surface charge it is reasonable to assume that charge is the main reason for their distribution.

3.4 DISCUSSION

In this chapter, investigations have been made on DNA and liposome binding to CAPT protoplasts, and, for comparison, with protoplasts of the normal (*i.e.* non tumorous) suspension cell culture of *N.sylvestris*, NS-1. Although several authors have reported binding and uptake of DNA in plant cells and protoplasts (*e.g.* Ohyama *et al.*, 1972; Uchimiya and Murashige, 1977; Hughes *et al.*, 1978), Behki and Lesley (1979) point out that the techniques used in such studies may provide inaccurate information. In particular, many authors have quantified the amount of DNA uptake as the amount of undegraded radioactive DNA in samples, following protoplast lysis. As illustrated by the work of Behki and Lesley (1979) much of this "apparent" internalised DNA may in fact have detached itself from the outer membrane and reattached to cytoplasm or cellular organelles during the process of protoplast lysis. An autoradiographic approach to studying the fate of foreign DNA in plant cells should circumvent this particular problem. However, the report of Kool and Pelcher (1978), in which autoradiographic techniques were

employed, found that no DNA bound to the surface of soybean protoplasts but only to whole cells of the culture. Some binding occurred in the protoplast suspensions but, on microscopic examination of autoradiographs, it was found that silver grains were associated with cell wall debris present in the suspension rather than with the protoplasts. Such an observation could not have been made using biochemical techniques.

Contrary to the results of Kool and Pelcher described above, the present autoradiographic study shows considerable levels of binding of CAPT DNA to CAPT protoplasts, and the work of Gould (personal communication) indicated similar levels of binding of CAPT DNA to NS-1 protoplasts. There seems to be little difference between a homologous (*i.e.* CAPT DNA and CAPT protoplasts) and a heterologous (*i.e.* CAPT DNA and NS-1 protoplasts) situation, which might suggest that the binding achieved is non-specific in nature. Binding of all 3 types of liposomes (negatively charged, neutral and positively charged) has also been achieved with CAPT protoplasts, and for negatively charged liposomes with NS-1 protoplasts.

Many authors have reported an increase in DNA uptake in the presence of polycations such as poly-L-ornithine and poly-L-lysine (Suzuki and Takebe, 1978; Fernandez *et al.*, 1978; Ohyama, 1978). The addition of copper or zinc ions further enhances DNA uptake in the presence of polycations. Behki and Lesley (1979) have presented data which indicates that the enhanced "uptake" of DNA is due to the formation of a complex between the DNA and the polycation, which protects the DNA from nuclease attack. Also, Faber *et al.*, (1975) suggest that the positively charged molecules of poly-L-ornithine or poly-L-lysine bind to the negatively charged surface of animal cells, thus allowing binding of the negatively charged DNA. Using electrophoretic techniques, Nagata and Melchers (1978) have shown that the overall surface charge on protoplasts of several species

is negative, so that the same type of electrostatic association would be expected to apply in plant systems. In agreement with previous reports in plants, DNA binding to CAPT protoplasts and NS-1 protoplasts (Gould, personal communication) requires the presence of a certain level (>3 µg/ml) of poly-L-ornithine. In addition, the binding of all 3 liposome types to both CAPT and NS-1 protoplasts requires similar levels of poly-L-ornithine. It was expected that positively charged liposomes might bind more readily to the protoplast surface in the absence of poly-L-ornithine. Giles (1978) has previously noticed more efficient binding of positively charged liposomes but no data on this aspect was presented in his report, so that it is not known if polycations were used to induce such binding. It may be that the positive charge on the liposomes is not displayed on the outer surface or is insufficient to produce attachment to the protoplast membrane. In the case of liposome binding, the hypothesis of the formation of a "protected" complex which has been put forward for DNA binding (Behki and Lesley, 1979) is unlikely to apply and electrostatic considerations are perhaps more likely to play a major role.

Work reported in this chapter has also approached the question of cell cycle related binding of DNA and liposomes to CAPT and NS-1 protoplasts. Initial studies on the cell cycle in CAPT protoplasts indicated that cycling stops during the enzyme treatment for protoplast isolation, so that cells which are pulse labelled with tritiated thymidine prior to enzyme treatment (these cells will be S-phase cells) should still be in S-phase immediately after removal of enzyme. It has been demonstrated that NS-1 cells similarly stop cycling during enzyme treatment (Gould, unpublished data). It is thus possible to distinguish S-phase protoplasts which will show nuclear labelling on autoradiographs, from the total protoplast population, and thus study the binding capacity of this particular

cell cycle stage as compared with the whole population. In addition, microspectrophotometry techniques have been used to measure the quantity of binding at different cell cycle stages and comparisons of binding, non-binding and total protoplast populations has generated information on the fractions of each cycle stage participating in binding.

Results of the analysis of the binding of CAPT DNA to CAPT protoplasts indicate that binding occurs more frequently to S-phase protoplasts (labelled population) than to the total protoplast population. Comparisons of the fractions of protoplasts in each cell cycle compartment for binding, non-binding, and total populations indicate that there is no enrichment for either G_1 or G_2 phase in the binding fraction. However, the amount of DNA binding (*i.e.* silver densities as measured by microspectrophotometry) is greater for G_2 phase protoplast than G_1 phase protoplasts. It is probable that this difference is related to size difference between G_1 and G_2 protoplasts, although it is noted that the increase in binding is less than the expected increase in surface area of G_2 protoplasts. It is difficult to make assumptions about the quantity of binding to S-phase protoplasts from this data since so few examples of this cycle stage are included in the analysis. These results, then indicate preferential binding to S-phase protoplasts, and, as such, are contrary to the results of Gould (unpublished data) on CAPT DNA binding to NS-1 protoplasts, which showed that fewer S-phase protoplasts bind DNA as compared with G_1 or G_2 phase protoplasts.

Results of the liposome binding experiments again point to a differential response of S-phase protoplasts. In particular, positively charged and neutral liposomes bind less fequently to S-phase (labelled) CAPT

protoplast populations. Equivalent data, using labelled populations, is not available for the binding of negatively charged liposomes or for NS-1 populations. However, microspectrophotemetry data for binding of negatively charged liposomes to NS-1 indicates that there are a reduced number of S-phase protoplasts in the binding population. CAPT protoplast populations binding these liposomes show less variation from the normal distribution as regards the frequency of S-phase protoplasts. It must be admitted that in either case, the low numbers of S-phase cells recorded in microspectrophotometric analysis make it difficult to draw firm conclusions from this data alone.

An additional phenomenon, noticed with liposome binding, but not observed with DNA binding is the greater number of G_2 phase protoplasts, and smaller number of G_1 phase protoplasts in the binding populations of both CAPT and NS-1 treated with negatively charged liposomes. In addition, the quantity of binding (*i.e.* silver density) to G_2 phase protoplasts is considerably greater than the binding to G_1 phase protoplasts for both CAPT and NS-1 treated with negatively charged liposomes. This increased quantity of binding is of the order of magnitude expected for the increased surface area of G_2 phase protoplasts. It is thus suggested that the increased fraction of G_2 phase protoplasts in the binding population is related to the increased capacity for binding at this cell cycle stage, which increases the chance of inclusion of these protoplasts in the binding population. Increased binding (approximately double) also occurs, in all cycle stages, for NS-1 protoplasts as compared with CAPT protoplasts. This species difference is obviously related to the size difference between NS-1 and CAPT protoplasts. Thus, size considerably affects the capacity for binding of negatively charged

liposomes, but it is noted that the increased surface area, and hence the increased membrane components, would be expected to produce an increased net surface charge, so that an electrostatic model for the mechanism of binding would still be supported by this data.

The cell cycle related data from these analyses of the binding of DNA and liposomes enables certain conclusions to be drawn. In particular, it has been noticed that S-phase protoplasts display differential binding capacity (a raised capacity in the case of DNA binding to CAPT protoplasts, and a lowered capacity for liposome binding to both CAPT and NS-1 protoplasts). This observation fits in with previous studies on the binding of Tobacco Mosaic Virus (TMV) to NS-1 protoplasts, where S-phase protoplasts show a reduced binding capacity (Gould *et al.*, in press). As previously mentioned, the DNA binding process is considered to be at least partly governed by electrostatic factors, and liposome binding may also be expected to rely on charge interactions. It is thus proposed that differential binding at different cell cycle stages is related to fluctuations in overall surface charge which may occur during cycle progression, as has been observed with animal cells (Brent and Ferrester, 1967). In particular, the reduced binding capacity at S-phase for NS-1 protoplasts with DNA, negatively charged liposomes, and TMV suggest that there is a reduced overall surface sharge at this DNA synthetic stage, which rises in both G_1 and G_2. The overall charge may be somewhat larger at G_2 than at G_1, as suggested by the increased capacity for binding at G_2. This is represented diagrammatically in Fig. 3.25.

This cyclic fluctuation in surface charge, associated with cell cycle stage, is perhaps supported by evidence obtained by Coutts and

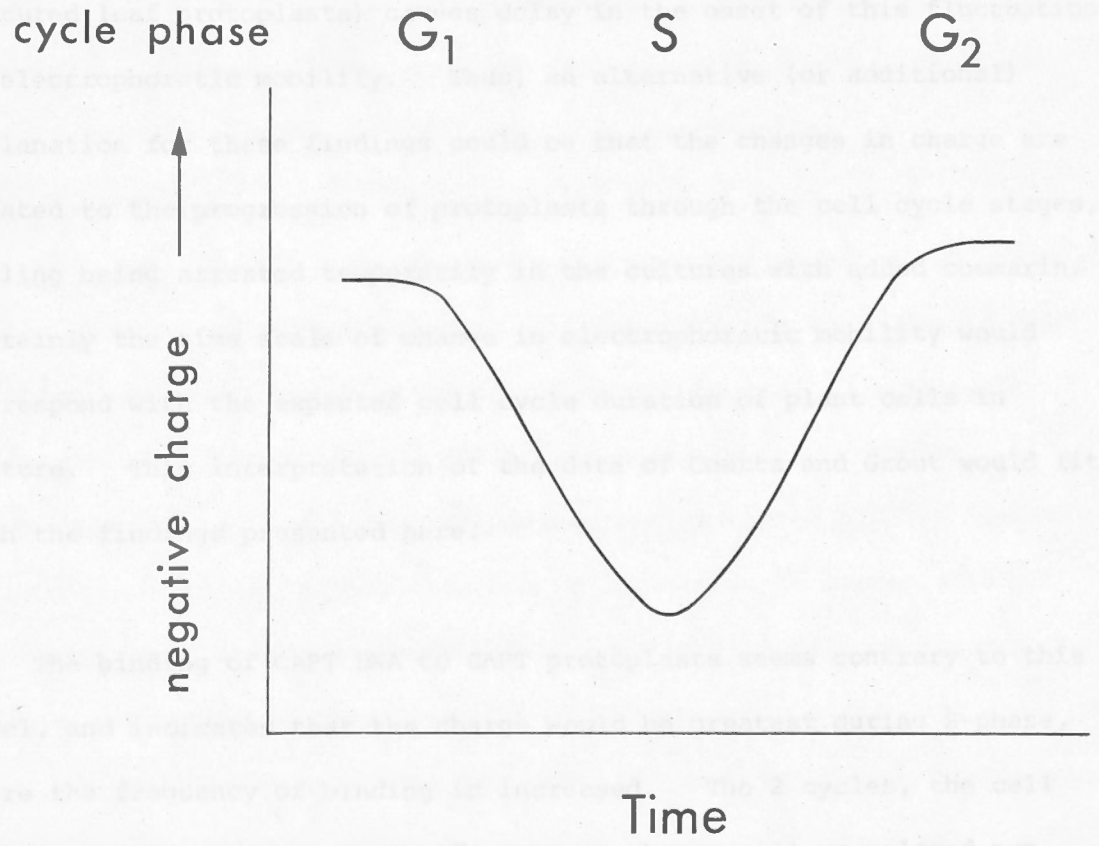


FIG. 3.25 Proposed model for the change in surface charge through the cell cycle.

Grout (1975) using leaf protoplasts of *Nicotiana tabacum*. They observed a cyclic fluctuation in surface charge during the initial stages of culture of the mesophyll protoplasts, and they proposed that this was related to events in the sequence of *de novo* cell wall regeneration. The addition of coumarin (which apparently delays the onset of division in cultured leaf protoplasts) causes delay in the onset of this fluctuation in electrophoretic mobility. Thus, an alternative (or additional) explanation for these findings could be that the changes in charge are related to the progression of protoplasts through the cell cycle stages, cycling being arrested temporarily in the cultures with added coumarin. Certainly the time scale of change in electrophoretic mobility would correspond with the expected cell cycle duration of plant cells in culture. This interpretation of the data of Coutts and Grout would fit with the findings presented here.

The binding of CAPT DNA to CAPT protoplasts seems contrary to this model, and indicates that the charge would be greatest during S-phase, where the frequency of binding is increased. The 2 cycles, the cell division cycle, and the cycle of membrane change will be related but may become separated in time so that it may be, for example, that the expected membrane change which causes a decreased charge at the DNA synthetic stage may be delayed somewhat, causing a high charge at S-phase. This may be a reasonable explanation for the preferential DNA binding to S-phase CAPT protoplasts. It is known that the dividing population of the CAPT culture progresses very rapidly through the G_1 phase (see Chapter 2, also Ashmore and Gould, 1979). It may be that the unusual kinetics of cell cycle progression displayed by the CAPT culture causes upset in the cycle of membrane change. For example, cells which progress

very rapidly through the G_1 phase into S-phase may still retain the higher charge of the G_1 phase during the DNA synthetic process. On the other hand, it may be that the tumorous nature of the CAPT culture affects the membrane properties such that they differ from normal cells. Certainly, alterations in surface properties, and particularly surface charge, apparently occur in animal tumour cells (Forrester *et al.*, 1962; Borysenko and Woods, 1979), and such deviations from normal membrane characteristics might also occur in plant tumour cells.

Both the DNA and liposome binding experiments required the presence of the polycation poly-L-ornithine, which apparently acts by attaching to the negatively charged surface of the protoplasts, thus allowing binding of negatively charged DNA or liposomes to the protoplasts. These experiments therefore provide information on the binding capacity of poly-L-ornithine, rather than DNA or liposomes, to protoplasts of different cycle stages. Nevertheless, the implications as regards the surface properties of protoplasts in different cycle stages are still the same. Also, the observations are still of value to an understanding of how to perform transformation experiments since polycations will presumably be required to allow the initial attachment in such experiments. It was noticed that positively charged and neutral liposomes bind less readily to S-phase CAPT protoplasts which is contrary to the observations on DNA binding to protoplasts of this culture. This phenomenon may be explained by the fact that the charge considerations will be different in these cases and perhaps gives further evidence that the binding process is indeed a charge based reaction.

In order to try and confirm the model of surface charge differences

in different cycle stages, the aqueous 2 polymer phase system was used to look for differential partition of NS-1 protoplasts (which should separate according to surface properties in such a phase system) of different cycle stages. The NS-1 culture was used initially since the CAPT population would probably yield complex data due to the inclusion of the "non-cycling" fraction of this culture. Also, the low DNA content of CAPT cells makes S-phase difficult to distinguish by Feulgen microspectrophotometry, combined with the fact that S-phase is short in duration and therefore only represents a small fraction of the population (although it has been shown that enrichment for protoplasts in S-phase does occur during protoplast isolation). Obviously a CAPT protoplast population should be analysed using a 2 polymer phase system but it may perhaps be necessary to use pre-labelled cells to allow recognition of S-phase protoplasts. Information gained from countercurrent distribution of NS-1 protoplasts indicates that S-phase protoplasts do in fact separate differently from G_1 and G_2 phase protoplasts and thus seem to differ in surface properties, probably surface charge, although hydrophobicity may play some part in the separation process. Thus, the proposed model of change in surface charge during cycle progression appears to be supported by this data.

Further work is required in this area and the use of the techniques presented here should provide rewarding results in the future. In particular, use of an automatic device for countercurrent distribution of protoplasts should achieve better separation. Once the protoplast population has been enriched for a particular cell cycle stage by countercurrent distribution, the polymers may be removed, leaving a viable population of protoplasts which may be more "competent" for DNA or

liposome uptake. Use of a flash labelled population of protoplasts for 2 polymer phase separation would provide an extra marker for identification of cell cycle stage of the enriched populations. It is noted that the protoplast isolation procedure in the CAPT culture already provides enrichment for S-phase protoplasts which are apparently more competent for DNA binding, as shown in these experiments. The CAPT culture may be a good choice as host material in transformation experiments, not only because of low chromosome number, but also since DNA binds more frequently at S-phase, and it may be expected that the integration of foreign DNA is most likely to occur at the DNA synthetic stage.

Finally, many experimenters have achieved little uptake of DNA in leaf protoplasts which are presumably in an "arrested" G_1 state. Observations of Kool and Pelcher (1978) on cell cultures indicate that cultures in the log phase of growth are more susceptible to DNA binding. Wetter and Kao (1980) have suggested that it may be necessary to use dividing populations for fusion studies since they propose that specific combinations of cell cycle stages may provide the most stable hybrid material. Similarly, there would appear to be a need to use protoplasts from rapidly dividing populations of tissue culture cells for successful DNA binding, and the present report further suggests, in line with the ideas of Wetter and Kao, the need to enrich for particular cycle stages within such dividing populations.

CHAPTER 4

ANALYSIS OF FUSION IN CAPT AND NS-1 PROTOPLAST POPULATIONS

4.1 INTRODUCTION

The process of somatic cell hybridisation is perhaps the most widely used technique in animal cell genetics. The hybrid cell es extensively used for mapping studies (Boone *et al.*, 1972) and, by fusing cells which contrast in some important property it becomes possible to ask questions about dominance of control processes, nucleocytoplasmic interactions, and the nature of differentiation. Similar problems may be approached using plant tissue culture although less progress has been made in this area with plant systems. Maybe much of this work would only serve to reiterate the findings in animal systems, but plant systems do provide the additional opportunity to study the effect of changes at the molecular level on the whole organism, by the process of whole plant regeneration from cell culture. Somatic hybridisation of animal cells has also been restricted to the use of tissue culture populations, where changes in a cell's characteristic properties may already have occurred *i.e.* apparent dedifferentiation takes place in the tissue culture environment. On the other hand, plant protoplasts may be obtained from a wider variety of sources, including differentiated tissue of the whole plant *e.g.* leaf, petal; from young meristematic cells; from haploid cells of the reproductive tissues; or from tissue culture systems which may be slowly dividing callus growths or rapidly dividing suspension cell cultures.

Several chemical fusogens have now been described (Lucy, 1978), including many lipid soluble substances such as lysophosphatidylcholine, and composite lipid fusogens in the form of small vesicles (liposomes). Particularly

important amongst the water-soluble chemicals that induce cell fusion is the polymer, poly (ethylene) gycol (PEG), which was first employed by Kao and Michayluk (1974) to fuse plant protoplasts. PEG is a highly effective fusogen, which has been shown to cause fusion between cells/protoplasts from widely different sources. In the report by Kao and Michayluk (1974), it was shown that in mixtures of *Vicia hajastana* and *Pisum sativum* protoplasts the frequencies of homoplasmic and heteroplasmic fusions approached the theoretical distribution, indicating the effectiveness of PEG in inducing fusion at random between different protoplast types. In fact, it is possible to fuse animal cells with plant protoplasts *e.g.* the fusion of human cells with *Haplopappus* protoplasts (Lima-de-Faria *et al.*, 1977), and the hybridisation of *Xenopus* cells with protoplasts of *Daucus carota* (Ward *et al.*, 1980). In the latter report, the intermixing of the membranes of the plant and animal sources occurred within 3 h of the initial fusion event, as shown using immunofluorescence to detect animal specific membrane components.

PEG has the general formula HO $CH_2(CH_2\text{-}O\text{-}CH_2)_n$ CH_2OH, and the presence of the ether linkages make the molecule slightly negative in polarity. It may be that the long chain of the PEG molecule acts as a molecular bridge between the surfaces of adjacent protoplasts, so that adhesion, and fusion, occur. Maggio *et al.* (1976) have suggested that a charge reaction may be involved. They have shown that PEG causes a marked reduction in the surface potential of monolayers of phosphatidyl choline and phosphatidyl ethanolamine, and if such a reduced potential occurs on the surface of protoplast plasmamembranes, extensive aggregation of protoplasts would be expected.

The use of liposomes to induce cell fusion has been studied more extensively recently. Liposomes offer the advantage that the fusing cells remain highly viable, whereas treatment of cells with high concentrations of PEG causes extensive cell death with prolonged treatment. Papahadjopoulos *et al.* (1973) found that negatively charged liposomes (containing phosphatidylcholine and phosphatidylserine) were most effective in inducing cell fusion, which indicates that a charge reaction may again be involved in the fusion process.

Although the fusion procedure is itself fairly simple and apparently successful between closely related and widely separated organisms, the field of plant somatic hybridisation faces problems related to the selection of hybrid cells, the cytological analysis of the hybrid genome, and the maintenance of stable hybrids in culture. The lack of genetic markers in plant systems has forced workers to use other methods of hybrid cell selection which include:-

(i) the use of differential drug sensitivities and media requirements of parental types *e.g.* Power *et al.* (1976); Power *et al.* (1977);

(ii) mechanical methods *e.g.* Gleba and Hoffmann (1978); and

(iii) the fusion of wild type protoplasts with albino protoplasts *e.g.* Cocking *et al.* (1977); Schieder (1978).

Perhaps the auxin autotrophic nature of tumorous cell lines *e.g.* the CAPT cell line, could be used as part of a selective system for hybrid cells. The use of such low chromosome number species as *Crepis capillaris*, *Haplopappus gracilis* and *Brachycome dichromosomatica* would certainly allow easier cytological identification of hybrid cells, and monitoring of any large structural changes in the hybrid genome.

The problems of the maintenance of a stable hybrid may be a biological phenomenon which cannot easily be avoided *e.g.* the unidirectional loss of crhomosomes of one parental type from hybrid cells is commonly observed in animal systems (Weiss and Green, 1967) and has also been noticed in some plant systems (Power *et al.*, 1975; Kao, 1977). Wetter and Kao (1980) have solved this problem to some extent, in their *Glycine max* - *Nicotiana glauca* hybrids, where extensive loss of *N.glauca* chromosomes occurs (Kao, 1977). When protoplasts of hybrid cells were "back fused" twice with *N.glauca* protoplasts a considerable increase in stability of *N.glauca* chromosomes occurred. It has been suggested by Wetter and Kao (1980) that the instability of hybrid cells may be due to incompatibility of the original fused cells as regards cell cycle state. For instance, it is known that in animal systems fusion of a mitotic cell with an interphase cell may cause premature chromosome condensation of the chromosomes of the non-mitotic cell (Johnson and Rao, 1970) which perhaps disrupts the stability of the affected genome. Wetter and Kao (1980) therefore suggest that there may be an "ideal combination" of cell cycle stages which would give rise to a stable hybrid. Such a possibility could be tested by fusion of populations in which every cell cycle stage is represented *e.g.* rapidly dividing cell cultures. Many previous fusion experiments, including those of Wetter and Kao (1980) have used protoplasts isolated from leaf material, in which all cells are presumably arrested in the G_1 stage of the cell cycle.

In summary, the composite cell lines described in this thesis may present a good choice for fusion studies for several reasons:-

(i) The tumorous, auxin-autotrophic nature of the CAPT cell line may provide part of a hybrid selective system.

(ii) The low chromosome numbers of these species allows easier identification of hybrid cells and monitoring of the hybrid karyotype.

(iii) The C-banding achieved on CAPT and HA-1 chromosomes, apart from allowing more accurate monitoring of hybrid cells, may provide markers which correlate with properties of the hybrid cells. In addition, C-banding allows recognition of any large structural chromosomal rearrangements which may occur in the hybrid genome.

(iv) The cell lines are all available in the form of rapidly dividing suspension cell cultures, so that protoplasts at all cell cycle stages may be obtained.

(v) Some success has been made in culturing CAPT protoplasts to the callus stage.

This chapter, then, reports work which has been directed towards producing hybridisation within and between low chromosome number species. Although attempts were made to use both the CAPT and HA-1 cell lines, for technical reasons only the experiments using CAPT protoplasts were successful. NS-1 protoplasts were also used, for comparative purposes. Both PEG and negatively charged liposomes have been used as fusogens, and, initially, the optimum conditions for PEG fusion of CAPT protoplasts were assessed.

In accordance with the ideas of producing an "ideal combination" for stable hybrid production, and the possibility that cell cycle stage may be an important factor, binucleates have been analysed using microspectrophotometry and autoradiography to assess the radomness of fusion as regards cell cycle stage. Any specificity for certain cell cycle combinations

during the initial fusion event may be detected in this way. Since the fusogenic actions of both PEG and liposomes may be charge related, and, as noted in the last chapter, surface charge on protoplasts may change with cell cycle stage, it may be that cell cycle stage affects a protoplast's capacity for participation in fusion events, or the type of protoplast that it will fuse with. Studies with animal cells have previously shown no preferential involvement in fusion of any one particular cycle stage, and cell cycle combinations in binucleates were random (Yamanaka and Okada, 1966; Johnson and Harris, 1969). However, in this work, with animal cells, fusion was induced using inactivated sendai virus which may differ in mode of action from either PEG or liposomes.

Comparison of the results of binucleate analysis using PEG and liposomes may allow some ideas to be developed as to the modes of action of these fusogens. In addition an analysis has been performed on NS-1 binucleates, induced using Concanavalin A (Con A). This fusogen may be expected to act in a different fashion to either PEG or liposomes since there are specific receptors for binding of Con A on the plasmalemma surface.

4.2 MATERIALS AND METHODS

4.2.1 PEG Fusion

Protoplasts of CAPT and NS-1 suspension cell cultures were isolated as described in the General Materials and Methods Section, washed and resuspended in Protoplast Washing Buffer (PWB) at approximately 5×10^5 protoplasts per ml. A drop of protoplast suspension was then placed on a microscope slide and left for 10 min to allow the protoplasts to settle and partially

adhere to the slide. A drop of 40% PEG (Fluka, m.w. 1550) in 20 mM $CaCl_2$ was added to the fusion mixture and left for 5 to 10 min. After this time, 5 to 15 volumes of high pH buffer (PWB with 20 mM $CaCl_2$ at pH 10) were added to the fusion mixture, left for 10 min, then allowed to run off the slide before fixing in 75% 3:1 ethanol:acetic acid in water with a final sorbitol concentration of 11% to 13%. Protoplasts were left in fixative for at least 24 h at 4°C, washed in 3:1 ethanol: acetic acid, then stained with Feulgen ready for microspectrophotometry.

Preliminary experiments were carried out to investigate the effects of different levels of PEG and calcium on the fusion of CAPT protoplasts.

4.2.2 Liposome Induced Fusion

Negatively charged liposomes, in PWB, were prepared as described in the Materials and Methods Section of Chapter 3. Fusion was carried out as with PEG, merely replacing PEG with liposomes in the presence of 20µg/ml poly-L-ornithine as the fusogen.

4.2.3 Concanavalin-A Induced Fusion

Concanavalin-A (N-[acetyl ^3H] acetylated) (Radiochemical Centre, Amersham, specific activity 35.6 Ci/m mol [1.32 TBq m mol^{-1}]), was dissolved in 0.01 M phosphate buffer at pH 7.0 to give an activity per ml of 50 µCi [1.85 M Bq]. A diluted sample (approximately 1 µCi) of this solution was added to 3 ml NS-1 protoplasts (approximately 5×10^5 per ml), and gently mixed during a 10 min. period. After 10 min, the protoplasts were pelleted by centrifugation for 5 min in a bench centrifuge. The pellet was then resuspended in 3:1 ethanol:acetic acid containing 13% sorbitol. Protoplasts were Feulgen stained and autoradiographs were prepared as previously described (General Materials and Methods Section).

4.2.4 Pulse Labelling and Autoradiography

Pulse-chase labelling was performed on certain of the cell populations used for fusion, prior to the addition of protoplast releasing enzyme. This was performed, as described in the Materials and Methods Section of Chapter 2, and allows the identification of S-phase protoplasts. Autoradiographs were prepared using stripping film, in the usual manner.

4.2.5 Analysis of Binucleates

DNA contents (and hence cell cycle state) of nuclei in binucleates, which result from PEG, liposome or Con A induced fusion, were determined on Feulgen stained preparations, as described in the General Materials and Methods Section. From DNA profiles of populations of binucleates and unfused protoplasts on the same slide, approximate fractions of protoplasts in each cycle stage (*i.e.* G_1; S; G_2 [very few mitotic figures were seen]) were determined for the 2 populations. Where pulse-chase labelling was performed, S-phase protoplasts were identified as those which were labelled. Comparison of the fused and unfused populations allows assessment of any preferential involvement of any particular cycle stage in fusion events (at least the formation of binucleates). Using the DNA contents, and hence cycle stages, of the fused population, the expected frequencies of each cell cycle combination (e.g. G_1/G_1; G_1/S; G_1/G_2 etc.) in the binucleate population can be determined for a situation where fusion occurs at random, with no specificity for the fusion of like cell cycle combinations. These expected values have been compared with the observed frequencies of each combination in the binucleate populations and χ^2 tests have been performed to assess the degree of closeness of fit of this "random" model of fusion.

These analyses have been performed on CAPT protoplast populations

which were fused with PEG, and NS-1 protoplast populations which were fused with PEG, liposomes and Con A.

4.3 RESULTS

4.3.1 PEG Fusion

Preliminary results on the fusion of CAPT protoplasts (i.e. to produce homokaryons) presented in Table 4.1, indicate that high levels of fusion are achieved at concentrations of PEG of 30% and over. Below this concentration of PEG virtually no clumping of protoplasts was seen. An increased PEG concentration of 40% or 50% produced little difference in the degree of fusion observed and 55% of protoplasts remained viable even after a 30 min treatment with 50% PEG. With an increased concentration of $CaCl_2$ in the 40% PEG fusion mixture (*i.e.* 30 mM rather than 3 mM), protoplasts appeared to be healthier than at the lower calcium level, and viability (as indicated by the exclusion of Evan's Blue dye) was in fact higher than at 3 mM $CaCl_2$. In the absence of calcium no fusion was seen after 30 min. The fusion procedure adopted, and described in the Materials and Methods Section, involves the use of 40% PEG in 30 mM $CaCl_2$ for 5 min. This fusion treatment was shown to be successful in inducing the formation of both homokaryons (*i.e.* CAPT/CAPT or NS-1/NS-1) or heterokaryons (*i.e.* CAPT/NS-1).

4.3.2 Binucleate Analyses

4.3.2.1 PEG Fusion

Tables 4.2 and 4.3 indicate approximate fractions of protoplasts in each cell cycle stage of the fused (*i.e.* binucleate) and unfused populations

TABLE 4.1

Treatment	Degree of Fusion after 30 min.	Viability at 30 min. (Evans Blue)	Degree of Fusion after 60 min.
3 mM $CaCl_2$:-			
Control	-	65%	+
10% PEG	-	64%	+
20% PEG	+	59%	+
30% PEG	+++	58%	+++
40% PEG	+++	57%	+++
50% PEG	+++	55%	+++
No $CaCl_2$:-			
40% PEG	-	65%	+
30 mM $CaCl_2$:-			
40% PEG	+++	88%	+++

\- no clumping
\+ small number of fusions observed.
+++ very heavy clumping

from 4 analyses for NS-1 and 2 for CAPT, respectively. Analyses A and C of NS-1 represent 2 different fusion experiments which necessarily involve different batches of protoplasts whereas analyses B(i) and B(ii) are obtained from 2 different slides prepared from the same protoplast batch. The 2 analyses on CAPT are from separate fusion experiments with different batches of protoplasts. It can be seen that the fused NS-1 populations

TABLE 4.2 - NS-1 PEG FUSION

Analysis	Population Type	Fractions in each cell cycle stage		
		G_1	S	G_2
A	fused (n = 156)	0.72	0.16	0.12
	unfused (n = 49)	0.63	0.10	0.27
	control (*i.e.* before PEG addition) (n = 99)	0.68	0.09	0.23
B(i)	fused (n = 122)	0.73	0.06	0.21
	unfused (n = 50)	0.66	0.08	0.26
B(ii)	fused (n = 142)	0.67	0.18	0.14
C	fused (n = 264)	0.70	0.13	0.16
mean of fused $\pm$ SEM		0.705$\pm$0.013	0.133$\pm$0.026	0.158$\pm$0.019
mean of unfused $\pm$ SEM		0.645$\pm$0.015	0.09 $\pm$0.01	0.265$\pm$0.005

TABLE 4.3 - CAPT - PEG FUSION

Analysis	Population Type	Fractions in each cell cycle stage		
		G_1	S	G_2
A	fused (n = 50)	0.68	0.08	0.24
	unfused (n = 102)	0.61	0.16	0.23
B	fused (n = 202)	0.68	0.13	0.19
	unfused (n - 101)	0.74	0.05	0.21

contain slightly larger fractions of both G_1 and S-phase protoplasts, and considerably smaller fractions of G_2 phase protoplasts, as compared with the unfused populations. Thus, G_2 protoplasts appear to participate less frequently in fusion events, causing the apparent "raised" fractions in G_1 and S. Pulse-chase labelling was performed on sample C and it can be seen that there is good agreement between S-phase fractions determined by labelling, in this analysis, and those fractions obtained by DNA content measurements in the other analyses (see Table 4.2).

The 2 separate analyses on CAPT protoplasts seem to be conflicting, but, since analysis A relies on only 25 binucleates, it may be most appropriate to consider only analysis B, where 100 binucleates have been measured. From this latter set of data, it can be seen that considerably more S-phase protoplasts and fewer G_1 phase protoplasts are present in the fused population, as compared with the unfused population. Thus, S-phase protoplasts may preferentially partake in fusion, as compared with other cycle stages. In this analysis pulse-chase labelling was not performed, so that fractions of S-phase protoplasts were determined by DNA content alone, and may thus be open to error. However, NS-1 data showed good agreement between S-phase identification by DNA content alone, or by the presence of label.

Figures 4.1 → 4.3 show examples of Feulgen stained NS-1 protoplasts populations which have undergone the PEG fusion treatment. In Fig. 4.1, 2 binucleate protoplasts are indicated. Only those protoplasts which have no visible cell membrane between 2 nuclei are considered to be binucleates (telophase nuclei are of course also excluded from the analysis). Whereas the binucleate in the centre of the picture appears to contain 2 nuclei in like

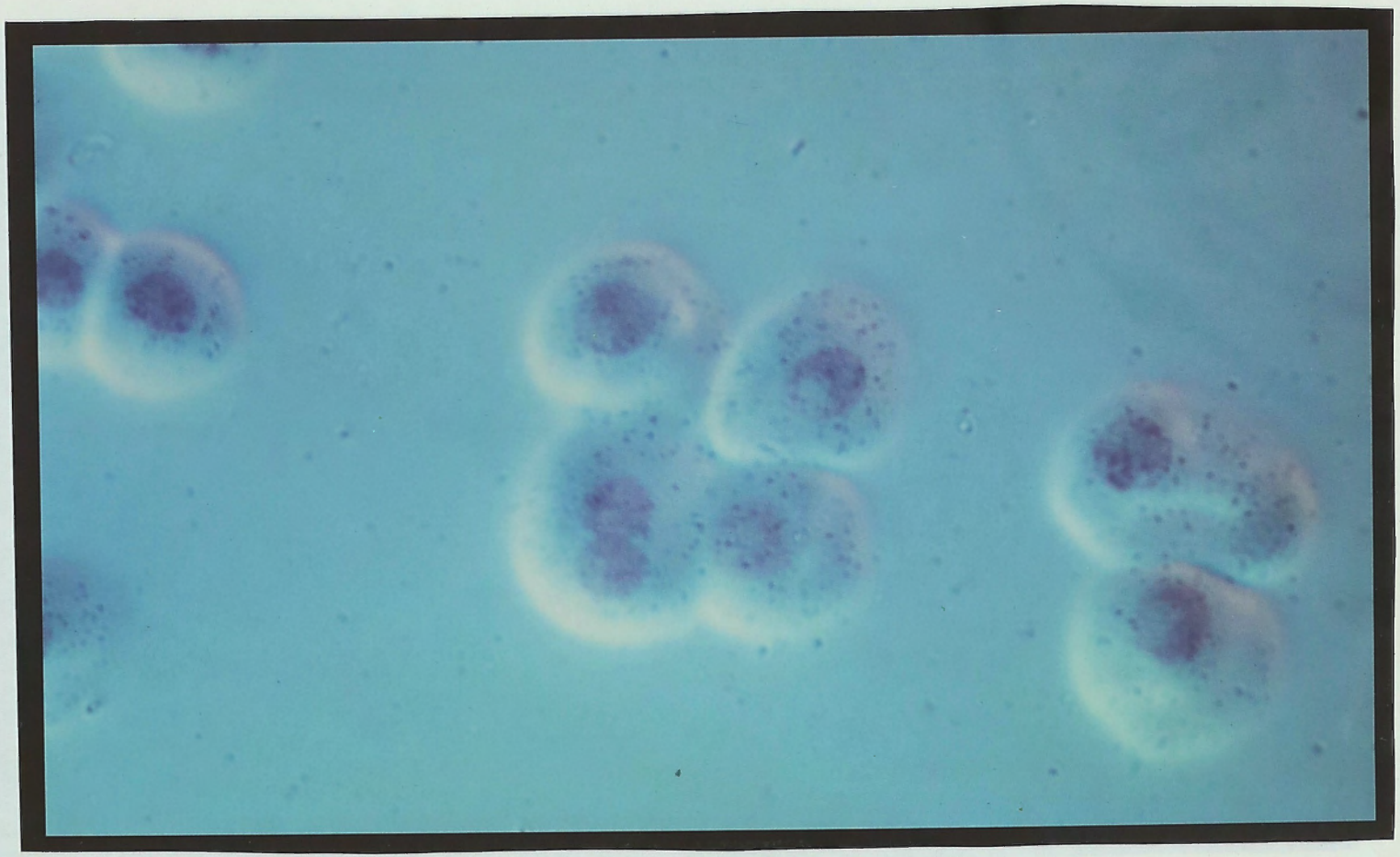


FIG. 4.1 PEG-fused NS-1 protoplasts with Feulgen stained nuclei.

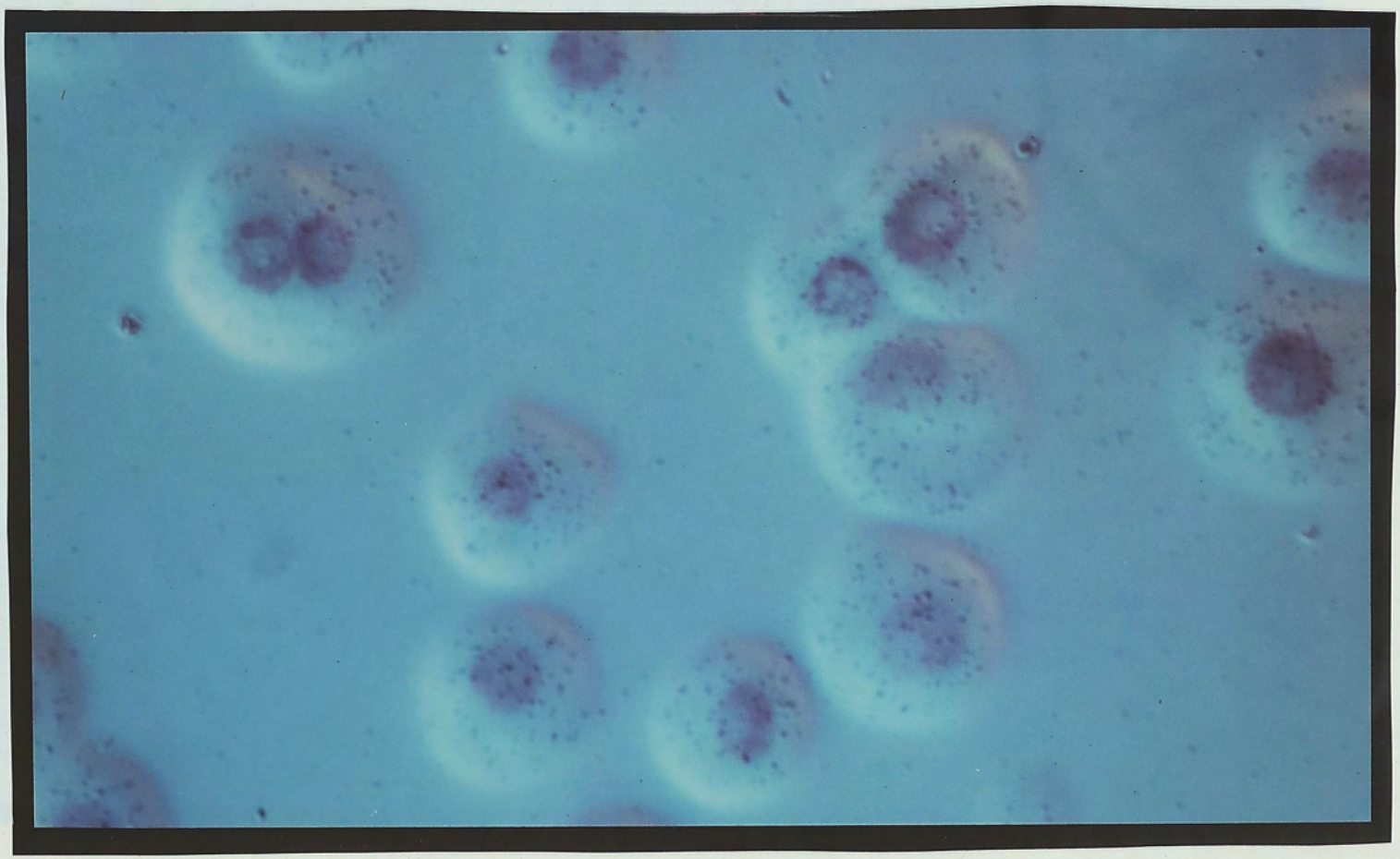


FIG. 4.2 PEG fused NS-1 protoplasts with Feulgen stained nuclei.

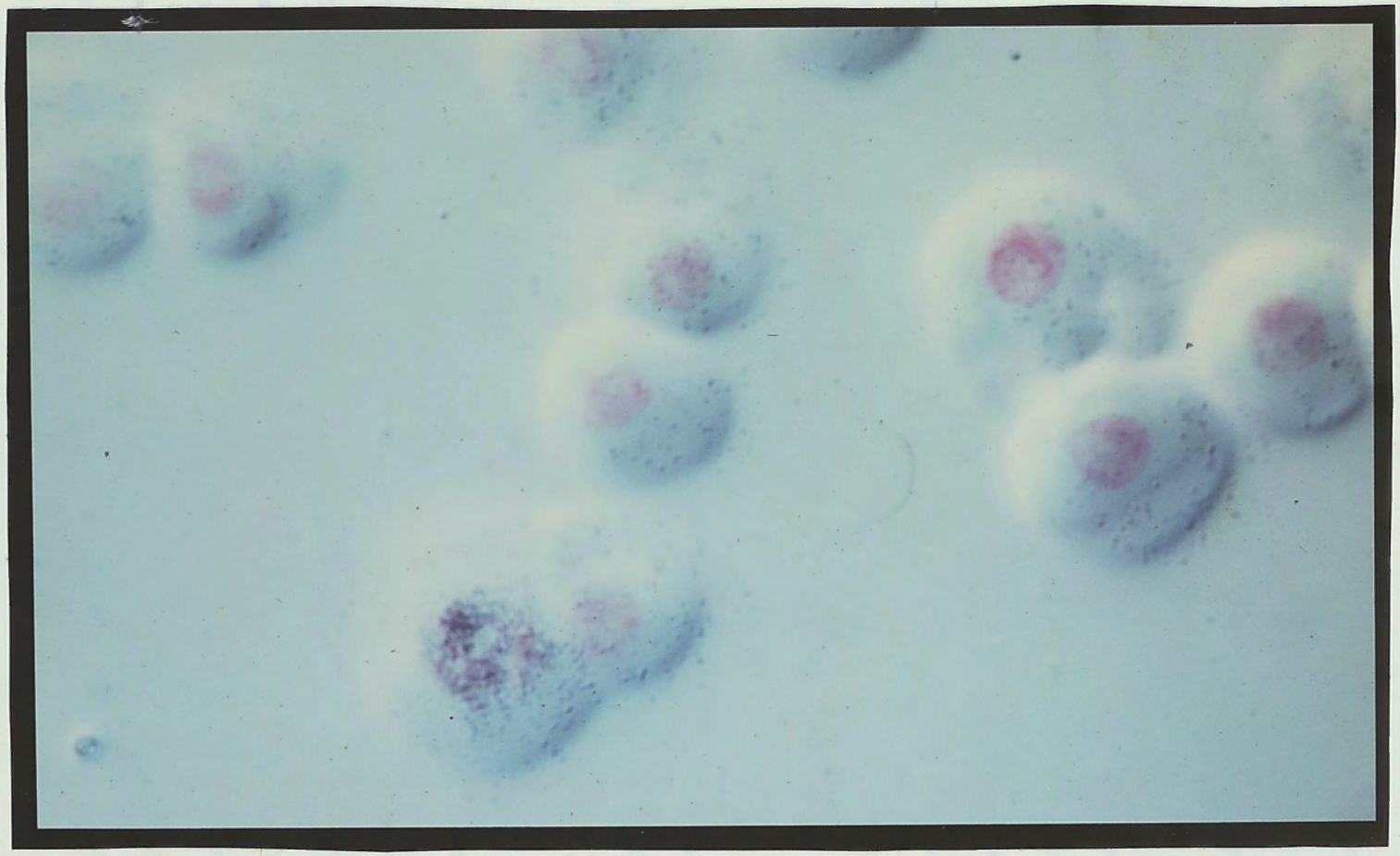


FIG. 4.3 PEG-fused NS-1 protoplasts with Feulgen stained nuclei. A binucleate with one labelled (*i.e.* S-phase) and one unlabelled nucleus is seen at the bottom of the figure.

cell cycle stages, probably both in G_1, the binucleate on the right hand of the photograph seems to contain 2 nuclei in unlike cycle stages, probably G_2 and S. In the case of the central binucleate the nuclei are touching, and perhaps beginning to fuse already only 5 to 10 min after the initial fusion event. Fig. 4.2 includes one binculeate in which the nuclei are probably both in G_1, and these nuclei are again touching, as if ready to fuse. Fig. 4.3 includes an example of a binucleate in which one nucleus is labelled (*i.e.* in S-phase) and the other is unlabelled and probably in G_1. Thus, it can be seen from these 3 photographs that both like cycle stages and unlike cycle stages participate in the formation of binucleates in various combinations, but it may be that nuclei in like cycle stages undergo fusion more readily once in a common cytoplasm. Confirmation of this latter observation would obviously require a much more detailed survey.

Binucleate analysis has allowed quantification of the expected and observed numbers of binucleates in like/like (*i.e.* G_1/G_1; S/S; G_2/G_2) and like/unlike (*i.e.* G_1/G_2; G_2/S; G_1/S) cycle stages, and, therefore, assessment of the degree of randomness of fusion with respect to nuclear cycle stage. χ^2 tests are shown in Table 4.4 and Table 4.5 for NS-1 and CAPT populations, and it can be seen that the cell cycle combinations G_2/S; G_2/G_2; S/S have been bulked in the analyses since expected values for these combinations each fall below 5. In all 4 separate analyses on NS-1, some deviation from absolute randomness occurs, analyses A and B(ii) deviating considerably more than the other 2 analyses. Analysis C is probably the most accurate, since pulse-chase labelling was used to identify S-phase protoplasts, and this gives a probability of between 0.1 and 0.5. Although absolute randomness is not observed, fusion between

TABLE 4.4 - NS-1 PEG FUSION

ANALYSES OF BINUCLEATES (including χ^2 tests)

Analysis	Cell Cycle Combination	Observed number	Expected number	Deviation	$\frac{\text{Deviation}^2}{\text{Expected}}$
A	G_1/G_1	47	40.4	6.6	1.08
	G_1/S	12	18.0	-6	2.0
	G_1/G_2	7	13.5	-6.5	3.13
	G_2/G_2	4	1.12	2.88	7.41
	G_2/S	3	2.99	0.01	0.003
	S/S	5	2.0	3	4.5
	*others	12	6.1	5.9	5.71
	$\chi^2(1.08 + 2.0 + 3.13 + 5.71) = 11.92$				
	with 3 d.f. p is close to 0.01 (11.34)				
B(i)	G_1/G_1	35	32.5	2.5	0.19
	G_1/S	3	5.34	-2.34	1.03
	G_1/G_2	16	18.7	-2.7	0.39
	G_2/G_2	4	2.69	1.31	0.64
	G_2/S	2	1.54	0.46	0.14
	S/S	1	0.22	0.78	2.77
	others	7	4.45	2.55	1.46
	$\chi^2(0.19 + 1.03 + 0.39 + 1.46) = 3.07$				
	with 3 d.f. p is between 0.1 (6.25) and 0.5 (2.37)				
B(ii)	G_1/G_1	39	31.87	7.13	1.59
	G_1/S	4	17.13	-13.13	10.06
	G_1/G_2	12	13.32	-1.32	0.13
	G_2/G_2	2	1.39	0.61	0.27
	G_2/S	4	3.58	0.42	0.05
	S/S	9	2.30	6.7	19.52
	others	16	8.68	7.32	6.17
	$\chi^2(1.59 + 10.06 + 0.13 + 6.17) = 17.95$				
	with 3 d.f. $p < 0.001$				

Continued

TABLE 4.4 - Continued

Analysis	Cell Cycle Combination	Observed number	Expected number	Deviation	$\frac{\text{Deviation}^2}{\text{Expected}}$
C	G_1/G_1	66	62.41	3.59	0.21
	G_1/S	18	22.91	-4.91	1.05
	G_1/G_2	8	10.27	-2.27	0.50
	G_2/G_2	5	4.21	0.79	0.15
	G_2/S	1	3.77	-2.77	2.04
	S/S	2	0.85	1.15	1.35
	others	8	4.41	3.59	2.92
	$\chi^2(0.21 + 1.05 + 0.5 + 2.92) = 4.68$				
	p is between 0.1 (6.25) and 0.5 (2.37)				

* For χ^2 tests, G_2/G_2; G_2/S and S/S have been grouped under "others" since expected values fall below 5 in each case.

TABLE 4.5 - CAPT PEG FUSION

ANALYSES OF BINUCLEATES (including χ^2 tests)

Analysis	Cell Cycle Combination	Observed number	Expected number	Deviation	$\frac{\text{Deviation}^2}{\text{Expected}}$
A	G_1/G_1	14	11.56	2.44	0.52
	G_1/S	5	8.16	-3.16	1.22
	G_1/G_2	1	2.72	-1.72	1.09
	G_2/G_2	3	1.44	1.56	1.69
	G_2/S	1	0.96	0.04	0.002
	S/S	1	0.16	0.84	4.41
	others	5	2.56	2.44	2.33
	$\chi^2(0.52 + 1.22 + 1.09 + 2.33) = 5.16$				
	with 3 d.f. p is between 0.1 (6.25) and 0.5 (2.37)				
B	G_1/G_1	52	46.7	5.3	0.60
	G_1/S	19	26.1	-7.1	1.93
	G_1/G_2	15	17.9	-2.9	0.47
	G_2/G_2	8	3.65	4.35	5.18
	G_2/S	4	4.99	-0.99	0.20
	S/S	3	1.71	1.29	0.97
	others	15	10.35	4.65	2.09
	$\chi^2(0.6 + 1.93 + 0.47 + 2.09) = 5.09$				
	with 3 d.f. p is between 0.1 (6.25) and 0.5 (2.37)				

cycle stages is certainly not specific. However, it is interesting to note that there are always more like/like combinations than expected (as indicated by the positive nature of the deviation in each of the analyses), and fewer like/unlike combinations than expected (as indicated by a negative deviation in each of the analyses, at least for G_1/S and G_1/G_2 combinations). Thus, although fusion between different cell cycle stages occurs in a close to random fashion, such deviation from randomness as exists, is directed towards like/like fusions.

In the analyses of CAPT binucleates (Table 4.5), a similar degree of deviation from absolute randomness occurs, again due to a tendency for like/like fusions to occur more frequently than expected.

It is noticeable that the category "others" in both the NS-1 and CAPT analyses contribute a large part of the χ^2 values in each analysis. However, removal of the value, and hence the loss of one degree of freedom makes little difference to the degrees of fit of the "random" model, in each analysis.

4.3.2.2 Liposome Fusion

Results of an analysis of liposome (negatively charged) induced fusion of NS-1 protoplasts are presented in Table 4.6. In this experiment, tritium labelled liposomes were used, and both the fused and unfused populations which have been measured include only those protoplasts which have some label (*i.e.* liposome) bound to the membrane, thereby excluding any fusions which might arise spontaneously. The approximate fractions (nuclear labelling was not used) of these populations in G_1; S; and G_2 phase have been estimated using microspectrophotometry. From this data, it appears that more G_1 phase protoplasts, and fewer G_2 phase protoplasts are present

TABLE 4.6 - NS-1 LIPOSOME FUSION

ANALYSIS OF FUSED AND UNFUSED POPULATIONS

Population	Fraction in each cell cycle stage		
	G_1	S	G_2
fused (n = 136)	0.48	0.16	0.36
unfused (n = 115)	0.38	0.16	0.46

BINUCLEATE ANALYSIS (including χ^2 test)

Cell Cycle Combination	Observed number	Expected number	Deviation	$\frac{\text{Deviation}^2}{\text{Expected}}$	Mean Silver density ± S.E.M.
G_1/G_1	24	15.67	8.33	4.43	14.08 ± 2.36
G_1/S	8	10.44	-2.44	0.57	12.88 ± 4.72
G_1/G_2	7	23.50	-16.50	11.59	15.29 ± 5.56
G_2/G_2	19	8.81	10.19	11.79	19.1 ± 4.12
G_2/S	4	7.83	-3.83	1.87	9 ± 3.63
S/S	6	1.74	4.26	10.43	39 ± 16.75

$\chi^2(4.43 + 0.57 + 11.59 + 11.79 + 1.87) = 30.25$

with 4 d.f. $p < 0.001$

in the fused population, as compared with the unfused population. Binucleate analysis is included in Table 4.6, and the χ^2 test has been performed using 5 categories of cell cycle combination (category S/S has been excluded from the test since the expected value falls below 5). It can be seen that this test indicates that there is a considerable deviation from randomness of fusion (as regards cell cycle combination), more so than with PEG induced fusion (see Tables 4.4 and 4.5). This deviation away from randomness appears to be due to like/like fusions occurring more commonly than expected, particularly G_2/G_2 fusions, and perhaps S/S fusions (this is less certain, due to smaller sample size).

The mean number of silver grains attached to binucleates varies considerably for each cell cycle combination as indicated by the large standard errors. However, there is perhaps an increased silver density (and hence liposome density) associated with G_2/G_2 type fusions (the increased silver associated with S/S type fusions again is uncertain due to small sample size).

It was also noted, in this study, that those binucleates which were seen to be undergoing nuclear fusions were always G_1/G_1 type fusions, suggesting that the capacity for nuclear fusion is perhaps increased at this cell cycle stage.

4.3.2.3 Concanavalin-A Fusion

Results of a binucleate analysis on Con A induced fusion in NS-1 protoplasts is presented in Table 4.7. There is a close fit between observed and expected numbers in each cell cycle combination, as indicated by the χ^2 test. Thus, fusion by Con A seems to be random in terms of cell cycle stage of protoplasts.

TABLE 4.7 - NS-1 CON A FUSION

ANALYSIS OF BINUCLEATES (including χ^2 test)

Cell Cycle Combination	Observed number	Expected number	Deviation	$\frac{\text{Deviation}^2}{\text{Expected}}$
G_1/G_1	30	27.56	2.44	0.22
G_1/S	18	19.68	-1.68	0.14
G_1/G_2	6	8.53	-2.53	0.75
G_2/G_2				
G_2/S				
others	10	8.22	1.78	0.39

$\chi^2(0.22 + 0.14 + 0.75 + 0.39) = 1.5$

p is between 0.5 (2.37) and 0.9 (0.584)

4.4 DISCUSSION

In this chapter results indicate successful fusion both within and between CAPT and NS-1 protoplast populations, to produce homo- and heterokaryons, using the chemical fusogen PEG. Also, fusion within NS-1 protoplast populations has been readily achieved using both liposomes and Con A. Both these latter fusogens have previously been employed in animal systems (Papahadjopoulos *et al.*, 1973; Halfer and Petrella, 1976), but this seems to be the first report in plant systems of the use of liposomes to induce protoplast fusion. Negatively charged liposomes (phosphatidylchloine : phosphatidylserine, 9:1) were used, in accordance with the results of Papahadjopoulos *et al.* (1974) which indicated effective fusion with liposomes of this type. In fact, more recent reports of Martin and Macdonald (1974 and 1976), and Lucy (1978) indicate that positively charged liposomes containing stearylamine or oleylamine may be

more effective in inducing fusion, oleylamine perhaps being more effective than stearylamine (at least with animal cells) due to its liquidity at incubation temperatures (Bruckdorfer *et al.*, 1974). The author has, in fact, looked at the binding of stearylamine containing liposomes to protoplasts (see Chapter 3) and found that poly-L-ornithine was required for binding, as with negatively charged liposomes. This suggests that positively charged liposomes react with protoplasts in the same way as negatively charged liposomes, but further studies should obviously be made into the use of positively charged liposomes (perhaps containing oleylamine) for fusion of plant protoplasts.

The studies of PEG fusion of CAPT protoplasts indicated the need for calcium in the fusion mixture. This is in agreement with previous observations on plant protoplast fusion (*e.g.* Kao and Michayluk, 1974) and animal cell fusion (see Lucy, 1978). The mechanism (or mechanisms) of action of calcium ions in the fusion process is unknown, although several possible explanations have been proposed. For example:-

(i) Calcium is known to cause lipid condensation (Papahadjopoulos, 1968) which produces a change in transition temperature of the liquid-gel transformation and may thus render membranes more fluid.

(ii) Calcium affects the orientation of the polar moieties of the membrane phospholipids which in turn would affect the interaction between membranes.

(iii) Calcium may merely restore stability of cell membranes once they have been broken and rejoined during the process of fusion (see Lucy, 1978; Gingell and Ginsberg, 1978).

As regards the binucleate analyses results indicate that in both PEG and liposome induced fusion of NS-1 protoplasts, G_1 phase protoplasts (and S phase protoplasts in the case of PEG fusion) participate more frequently than other cycle stages, and in the case of PEG induced fusion of CAPT protoplasts, S-phase protoplasts participate most frequently. As far as the specifity of fusion, in terms of cell cycle stage, is concerned, it was found that with both PEG and liposome induced fusion, some deviation from randomness occurred, particularly with liposome induced fusion. Con A induced fusion of NS-1 protoplasts was random in terms of cell cycle combinations.

Thus, cell cycle state appears to affect the ability of the protoplasts to participate in fusion *i.e.* G_1 and S phase protoplasts seem to participate more readily than G_2 phase protoplasts. As mentioned in the introduction to this chapter, the fusogenic action of PEG is most likely to be associated with its ability to reduce the normally negative surface potential of cells, thereby allowing cells to come into close contact with one another (Maggio *et al.*, 1976). Lucy (1978) has noted that the fusogenic lipids give rise only to a rather small decrease in mean surface potential per molecule when they interact with monolayers of phosphatidycholine (Magio and Lucy, 1976) whereas PEG, which is much more effective in inducing cell fusion causes a large decrease in surface potential. These observations certainly seem to indicate that a decrease in the surface potential of biological membranes may be of major importance in the process of membrane fusion. Studies with plant protoplasts (rather than with lipid monolayers) also indicate that fusogenic agents cause a reduction in surface potential. Using electrophoretic techniques a reduced surface potential was noted on leaf protoplasts of *Zea mays* in the presence of PEG and Ca^{2+} (Halim and

Pearce, 1980), and on *Nicotiana tabacum* protoplasts in the presence of Con A (Grout and Coutts, 1974). In the case of liposome induced fusion of NS-1 protoplasts reported here, poly-L-ornithine was present in the fusion mixture, and Grout and Coutts (1974) have also confirmed that poly-L-ornithine similarly reduces the negative potential on the surface of plant protoplasts from various sources. Thus, with all 3 types of fusion reported here, fusogenic action may be associated with a charge reduction on the plant protoplast plasmalemma.

In Chapter 3 it was concluded that surface charge may fluctuate with cell cycle stage, so that it may be expected that cell cycle stage will affect the fusion process. The results presented here have indicated that fewer G_2 phase protoplasts (at least for the NS-1 populations) participate in fusion than may be expected. This result would be in line with the fact that the surface potential is apparently largest at G_2 phase, thus requiring more reduction in charge before fusion can occur. Similarly, S-phase protoplasts may be expected to partake in fusion events more readily since the surface potential may already be lower at this stage (see Chapter 3). As expected, S-phase protoplasts are in fact represented in larger numbers in both NS-1 and CAPT PEG induced binucleates.

Having found that there may be similarities in the mechanism of fusion of all 3 fusogens used (PEG, liposomes and Con A), it remains to suggest why the binucleate analyses indicate differing degress of randomness of fusion as regards cell cycle stage. Despite the fact that a charge-based reaction may be involved in each case, it may be that the 3 fusogens vary in the extent to which they promote reduced surface potential (as noted by Lucy for lipid fusogens), and thus the extent to which a charge reaction,

and cell cycle effects will be involved. Additionally, the mechanism of cell fusion may depend on several factors *e.g.* membrane fluidity or membrane composition, the charge reaction being just one factor in the overall fusion process. Con A would certainly be expected to act differently from the other 2 fusogens since specific receptors for Con A occur on membrane surfaces. Thus, Con A may be expected to bind to the protoplasts and effect fusion in this way. In the case of PEG fusion, Gould (unpublished data) has shown that labelled PEG does not directly bind to NS-1 protoplasts and most likely causes fusion by merely bringing protoplasts closer together. Liposomes certainly bind to protoplasts in the presence of poly-L-ornithine, but, in this case, binding will most likely be due to a charge-based reaction whereas Con A binding is probably a more specific adhesion process. The fact that there are noticeably more G_2/G_2 binucleates than expected with liposome induced fusion is in line with the idea that binding is directly involved, since the results of Chapter 3 indicate that more liposome binding occurs with G_2 protoplasts than with protoplasts of other cycle stages. Thus, differences in the binucleates analyses for all 3 types of fusogen may be explained in terms of the mechanism of fusion in each case. The fact that more like/like combinations occur with liposome induced fusion, where the process of binucleate formation is most likely to be based on strong charge association, which actually involves binding of liposomes to the surface of the plasmalemma, tends to support the ideas of charge variation during the cell cycle.

Since all possible cycle combinations have been obtained in each of these fusion studies, investigations could be carried out to determine which, if any, of these combinations might produce the most stable hybrid cell line, in accordance with the ideas expressed by Wetter and Kao (1980). Certainly, it allows entrainment phenomena which have been observed in

some plant systems, to be studied in detail. Such work was carried out with animal systems several years ago (Yamanaka and Okado, 1966; Johnson and Harris, 1969). On the other hand, the fact that certain combinations of cycle stage may be more readily obtained using particular fusogens may also be exploited. For instance, in order to enrich for G_2/G_2 binucleates, fusion might best be carried out using liposomes. Alternatively, if a sample of unlike combinations is required, this could maximally be achieved using Con A fusion. Such unlike combinations may be more likely to lose parts of the hybrid genome through disruptions of the division process, and may thus be useful for genetic mapping studies.

The fact that some degree of specific fusion occurs, as regards cell cycle stage, is of interest in itself, and indicates that a cell's nuclear stage (*i.e.* cell cycle stage) may be recognised, to some extent, via the cell membrane. A similar idea, coined by the term "membrane impression", suggests that, during the process of cytodifferentiation, gene sets, which become expressed at different stages of a cell's history, code for particular sets of receptors in the membrane, such receptors being important for cell/cell associations (Brunner, 1977). It may be that the cell's nuclear state similarly affects the nature of the cell membrane.

GENERAL DISCUSSION

This thesis has been directed towards assessing the use of low chromosome number composites for studies at the cellular level, particularly for fusion and uptake studies. Initial investigations were made in order to describe certain characteristics of the tissue culture lines derived from the 3 species *Crepis capillaris*, *Haplopappus gracilis* and *Brachycome dichromosomatica*, namely the chromosome constitutions and cell cycle characteristics of the populations. It was felt that such initial detailed knowledge of cultures to be used in manipulation experiments would aid in later analysis of such experiments.

As isolated pieces of work, both the chromosomal studies of Chapter 1, and the cell cycle studies presented in Chapter 2, have produced new information and ideas which have contributed to the relevant fields. The chromosome analysis is the first such detailed study of 3 closely related species, in tissue culture, and the low chromosome numbers have allowed such analyses to be performed with relative ease and accuracy. Through continuous monitoring of the chromosomal constitutions of the cultures, it has been possible to propose a common pathway of karyotypic evolution in the tissue cultures of these three species. This pattern of chromosome change involves initial tetraploidisation followed by chromosome loss and rearrangements to produce a stable aneuploid chromosome set. Such a pathway of karyotype evolution has previously been proposed in plant culture systems (Bayliss and Gould, 1974), although without the more specific chromosome data presented here. Such a tetraploidisation-segregation pathway of chromosome change has long been accepted as a general pattern of karyotype evolution in many animal cell cultures (Levan and Biesele, 1958; Terzi and Hawkins, 1975), and it remains to be seen whether further detailed chromosome analsyes in

plant systems would confirm such a pattern of change in other plant tissue cultures.

The further use, in this chromosomal study, of the Giemsa C-banding technique, which has only been used, rather unsuccessfully, in 2 other plant tissue culture systems (Papeš *et al.*, 1978; Wochok *et al.*, 1980) has proved to be very valuable in ascertaining any chromosomal rearrangements within the studied genomes. In the case of the tumorous suspension cell culture of *Crepis capillaris*, CAPT, extensive rearrangements were revealed by the banding method (Ashmore and Gould, in press). Such considerable restructuring of the genome has not previously been observed in other plant tissue culture systems and opens up the possibility that either:-

(i) such rearrangements have remained unnoticed in other plant culture systems since banding techniques have not been employed, or

(ii) structural rearrangements are particularly prevalent in plant tumours, perhaps due to the integration of the Ti plasmid into the genome.

Such possibilities obviously require further investigation. It is also to be hoped that further use will be made, in the future, of banding techniques in plant tissue cultures to aid in more accurate chromosome analysis, particularly where chromosomes within a genome may otherwise be difficult to distinguish by gross morphology alone. Certainly, where experimental plans include attempts at genetic manipulation, it would be preferable to have performed a detailed chromosome analysis on the "host" genome prior to experimentation. As observed in the HA-1 suspension cell culture, what appears, at first sight, to be, say, a true diploid or true tetraploid may in fact contain "hidden" rearrangements which may

be visualised using banding methods. Banded chromosomes may additionally provide markers for use in transformation and hybridisation studies.

As regards the cell cycle studies, the analysis of the CAPT suspension cell culture has proved to be particularly interesting. This appears to be the first report on cell division kinetics in a tumorous plant population, which is surprising since unusual division properties are an important aspect of tumorous cells. The resultant model for cell kinetics in the CAPT culture, which has been produced using data from several different analyses, indicates that there is a low growth fraction in the population, the arrested cells apparently being in the G_1 phase, and that those cells which cycle have either no G_1 or a G_1 phase of very short duration (Ashmore and Gould, 1979). The latter observation is contrary to other studies of the cell cycle in plant tissue cultures, where G_1 phase has been shown to be of extended duration (*e.g.* Gould, 1977). The features of low growth fraction and shortened G_1 phase have often been observed in animal tumours, but further work is necessary to reveal whether these aspects of cell division kinetics are common to other plant tumour populations. This study has highlighted the need for a multiple approach to cell cycle studies since one type of analysis (*e.g.* FLM curve), applied in isolation, would have provided an incorrect picture of the division kinetics in the CAPT population. As regards the use of this culture for further studies on uptake and fusion, it is useful to know that a fraction of the population will be in an "arrested" state, and may therefore be unsuitable for such studies. It is worth noting that the apparent lack of G_1 in cycling cells of the CAPT culture may allow synchrony to be more readily maintained since populations are most likely to develop asynchrony during the G_1 phase.

The third aspect of the work in this thesis has included both DNA and liposome binding to CAPT and NS-1 protoplasts and the fusion of these protoplasts using 3 different chemical fusogens. The NS-1 cell line has been used as an example of a non-tumorous line for comparison with the tumorous CAPT cell line. The tobacco cell line was particularly chosen for these studies because it was readily grown in suspension cell culture and protoplast isolation procedures, worked out in this laboratory were readily repeatable. Of course, cell cultures of *H.gracilis* and *B.dichromosomatica* could certainly be used for such experiments, but some difficulty was encountered with the repeatability of HA-1 protoplast isolation, and the *Brachycome* cell lines are, as yet, too lumpy to allow protoplast isolation to be readily achieved. These mentioned difficulties should be overcome quite easily, but lack of time precluded the present author from investigating them.

Both the binding and fusion investigations were looked at from a cytological and kinetic view, which, in itself, is a somewhat refreshing approach to such plant tissue culture work. Also, analysis relied heavily on the automated quantitative autoradiography method developed by Gould (1979) in this laboratory, which certainly has not been used, as yet, by other authors. By applying these methods it has been possible to visualise the binding of both DNA and liposomes to protoplasts. Previous reports of DNA binding/uptake had produced equivocal data, and the one autoradiographic study (Kook and Pelcher, 1978) indicated that there was no label attached to the protoplasts. As regards liposome binding, it was found that all 3 types of liposome (negative, neutral and positive) bound to CAPT and NS-1 protoplasts, in the presence of poly-L-ornithine. Some mention has previously been made of the possible use of liposomes for the introduction of foreign genetic information into plant cells

(*e.g.* Giles, 1978), but no substantial attempt at binding/uptake of liposomes to plant protoplasts has yet appeared in the literature.

The investigations of cell cycle related differences in DNA and liposome binding have produce interesting results. Based on the idea that binding in the presence of poly-L-ornithine is, at least partly governed by charge interactions, a model of the variation in surface charge through the cell cycle has been proposed (Fig. 3.25). This model has been supported by data from the 2-polymer phase separation of NS-1 protoplasts, which separates particles/cells according to surface properties. The CAPT protoplast population in fact indicates a greater frequency of DNA binding at S-phase, whereas the reverse was true for the NS-1 population. This has been explained, in terms of the model of surface charge variations, by the unusual kinetics of division already described in the CAPT culture. Whereas the lowered binding of DNA at S-phase in the NS-1 protoplasts is perhaps unfortunate since this is the stage (*i.e.* DNA synthetic stage) when integration of DNA into the genome is most likely to occur, the fact that the reverse might be true for the CAPT population opens up the possibility that tumorous populations may be more effective for use in transformation attempts. This remains to be tested with both the CAPT population and other tumorous cultures.

Attempts at fusion have indicated that liposomes may be used to induce plant cell fusion. Analysis of binucleates resulting from treatment with 3 different fusogens has revealed some differences in:-

(i) participation of different cell cycle stages in binucleate formation, and

(ii) the randomness of cell cycle combinations in binucleates.

This has produced ideas about the possible differences in the modes of action of the 3 fusogens, and has revealed that there is a tendency towards

the production of like/like cell cycle combinations, particularly with liposome induced fusion. Certainly, the use of such microspectrophotometry techniques used here will allow further investigation of entrainment phenomena, and the use of rapidly dividing cultures for fusion opens up the way for assessment of different cell cycle combinations in the production of stable hybrids.

In conclusion, this study has emphasized a quantitative cytological approach to the careful description of:-

(i) the chosen experimental systems (i.e. plant tissue cultures) in terms of their suitability as model "hosts" for foreign genetic material, and

(ii) the cellular processes involved in the first stages of interaction between (a) host plant cells (protoplasts) and possible genetic engeneering vectors (DNA and liposomes) and (b) between protoplasts during fussion.

It is encouraging to note that leading workers in the field of plant tissue culture and genetic manipulation have recently begun to stress that the biochemical methods traditionally used to analyse protoplast fusion or transformation experiments rarely allow accurate quantitation at the single cell level. In terms of protoplast fusion, Wetter and Kao (1980) have expressed interest in cell cycle effects, and Lurquin (1981) has specifically stated that cytological (especially autoradiographic) methods are preferable to biochemical analyses. Hopefully, the productive methods reported in this thesis can make a contribution to this shift in experimental approach.

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APPENDIX

Copies of 3 papers published during the time of this thesis work are enclosed in an envelope attached to the back cover.

Thesis corrections

Throughout the thesis:

(1) where 'data' appears, read verb in the plural.

(2) the word 'innoculum' should read 'inoculum'.

Sarah Ashmore.

SARAH ASHMORE

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PROTOPLASMA

Karyotype Evolution in a Tumour Derived Plant Tissue Culture Analysed by Giemsa C-Banding

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Summary

A hyperdiploid aneuploid karyotype, consisting of 7 chromosomes, has been found in a tumorous suspension cell culture of *Crepis capillaris* (2 n = 6). Giemsa C-banding has revealed that these 7 chromosomes show consistent patterns of differential staining in all dividing cells. This stable karyotypic situation has persisted during 18 months of cytological monitoring of the culture. Comparison with the diploid C-banded complement of the root tip indicates that numerous structural rearrangements must have occurred during the formation of the aneuploid complement. A likely pathway for evolution of this karyotype involves initial tetraploidy followed by chromosome loss. Such a mechanism has previously been proposed for a plant tissue culture system (BAYLISS and GOULD 1974) and commonly occurs in animal systems, particularly in animal tumours (TERZI and HAWKINS 1975). An alternative mechanism, which does not necessarily involve tetraploidy, is also proposed.

Keywords: C-banding; Chromosome instability; *Crepis capillaris;* Plant cell culture; Tumour.

1. Introduction

Deviations from the original diploid chromosome complement commonly occur during the long term culture of both plant and animal cell populations (HSU 1961, SUNDERLAND 1973, BAYLISS 1980). Numerical and structural variation in karyotype are particularly marked in malignant animal cell populations (FORD 1964). Similarly, SACRISTÁN and WENDT-GALLITELLI (1973) have shown much greater karyotypic variability in crown gall (tumour derived) callus cultures of *Crepis capillaris* (2 n = 6), as compared with those cultures derived from normal tissue of this species. Long term analyses of these tumorous plant cell lines reveal that, whereas the cultures show marked heterogeneity of karyotype during their first year, after a further 3 years

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the majority of cells in such cultured populations have a single characteristic, though abnormal, chromosome complement (Sacristán and Wendt-Gallitelli 1973, Sacristán 1975), indicating analogies with the stemline concept of animal tumors (Makino 1957).

The application of chromosome banding techniques to animal cell cultures has, in many cases, revealed a greater degree of chromosome heterogeneity than was previously suspected (Tiepolo and Zuffardi 1973, Hashmi *et al.* 1974). Surprisingly, however, no substantial attempt has been made to use this approach in the analysis of plant tissue cultures.

In the present study the Giemsa C-banding technique has been used to analyse the chromosome complement of a tumour derived suspension cell culture of *Crepis capillaris* (conveniently referred to as CAPT), and comparison has been made with the normal C-banded karyotype of the whole plant.

2. Materials and Methods

2.1. Cell Culturing and Growth of Root Tips

The CAPT cell line used in this study is derived from a callus culture of an *Agrobacterium* induced crown gall tumour of *Crepis capillaris* (Sacristán and Wendt-Gallitelli 1973), and has been kept in liquid suspension in B 5 medium without added hormones for over 4 years. The culture has been maintained by regular transfers to fresh medium every 7 days, as previously described (Ashmore and Gould 1979).

Seeds of *Crepis capillaris* (L.) Wallr. (supplied by the Botanic Gardens, Berlin), were germinated in the light at 22 °C on wet filter paper. The growing root tips were excised from 4- to 5-day-old seedlings for use in cytological analysis.

2.2. Metaphase Arrest and Preparation of Slides for Staining

The CAPT cells, and root tips of *Crepis capillaris* were incubated in 0.1% colchicine for 3 hours prior to fixation in 3 : 1 ethanol : acetic acid. After at least 24-hour-fixation, cells were softened in 45% acetic acid for one hour before being tapped out gently onto a subbed microscope slide, and squashed under a coverslip. The coverslip was removed after freezing on dry ice, and the slide was then washed in distilled water and left to dry for at least 24 hours.

2.3. C-Banding

Slides were treated with 0.2 N HCl for 20 minutes, then washed in distilled water prior to incubation in a saturated barium hydroxide solution for 2 minutes at 45 °C. Immediately after removal from the barium hydroxide solution, the slides were again washed in distilled water and then left in 2 × SSC for 60 minutes at 65 °C. After a final wash in distilled water, the slides were stained in 10% Giemsa in phosphate buffer (pH 6.8) for 5 to 10 minutes. All slides were permanently mounted in XAM (Gurr neutral mountant).

3. Results

The application of the C-banding technique to both root tips and cultured cells gave reproducible patterns of differential staining along the metaphase chromosomes. The full C-banded karyotype of a dividing root tip cell of *Crepis capillaris* is shown in Fig. 1 *a*. The complement consists of three chro-

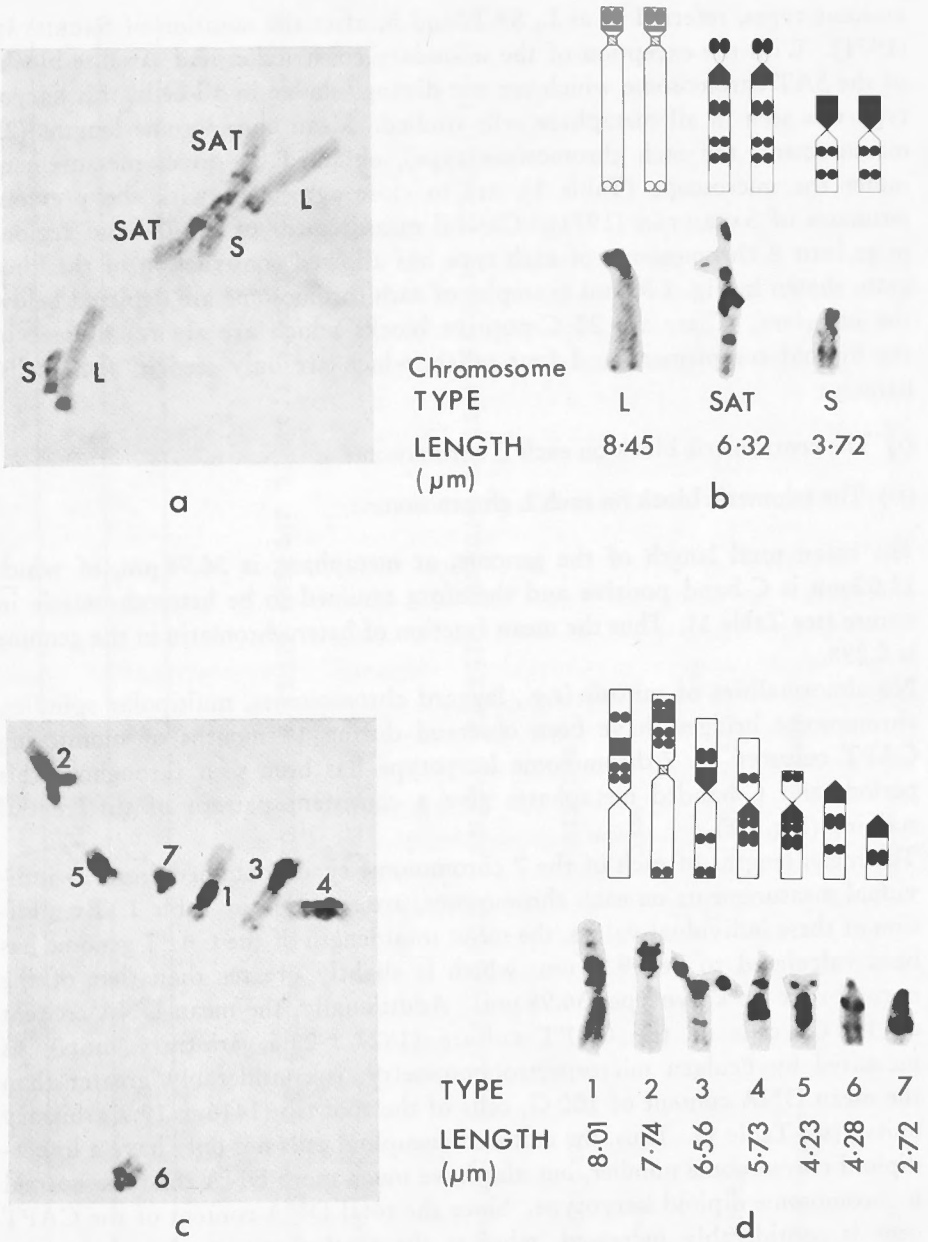

Fig. 1. *a* C-banded metaphase of a *Crepis capillaris* root tip cell. *b* Idiogram of the C-banded karyotype of *Crepis capillaris* root tip. Below the idiogram, representative examples of each chromosome type are shown, along with mean lengths, in micrometres of 8 to 10 chromosomes of each type. *c* C-banded metaphase of a CAPT cell. *d* Idiogram of the C-banded karyotype of the CAPT cell line. Below the idiogram, representative examples of each chromosome type are shown along with mean lengths, in micrometres, of 10 chromosomes of each type

mosome types, referred to as L, SAT, and S, after the notation of Sacristán (1971). With the exception of the secondary constriction and satellite blocks of the SAT chromosome which are not distinguishable in all cells, this karyotype was seen in all metaphase cells studied. Mean chromosome lengths (25 measurements for each chromosome type), obtained by direct measurement under the microscope (Table 1), are in close agreement with the previous estimates of Sacristán (1971). Careful measurement of the C-band regions in at least 8 chromosomes of each type has allowed construction of the idiogram shown in Fig. 1 *b*, and examples of each chromosome are depicted below the idiogram. There are 20 C-positive blocks which are always present in the diploid complement and four others which are only seen in some cells, namely:

(i) The centromeric block on each L chromosome

(ii) The telomeric block on each L chromosome.

The mean total length of the genome, at metaphase, is 36.98 μm, of which 11.02 μm is C-band positive and therefore assumed to be heterochromatic in nature (see Table 1). Thus the mean fraction of heterochromatin in the genome is 0.298.

No abnormalities of mitosis (*e.g.*, laggard chromosomes, multipolar spindles, chromosome bridges) have been observed during 18 months of monitoring CAPT cultures. A 7-chromosome karyotype has been seen throughout this period, and C-banded metaphases give a consistent pattern of differential staining (Fig. 1 *c*).

The mean lengths of each of the 7 chromosome types, obtained from 25 individual measurements on each chromosome, are recorded in Table 1. By addition of these individual values, the mean total length of the CAPT genome has been calculated to be 39.24 μm, which is slightly greater than that of the normal root tip karyotype (36.98 μm). Additionally, the mean DNA content of 100 G_1 cells of the CAPT culture (1827 ± 28.5, arbitrary units), as measured by Feulgen microspectrophotometry, is considerably greater than the mean DNA content of 100 G_1 cells of the root tip (1416 ± 19.2 arbitrary units), (see Table 1). Thus, the cultured aneuploid cells not only have a hyperdiploid chromosome number, but also have much more DNA than the normal 6-chromosome diploid karyotype. Since the total DNA content of the CAPT cells is considerably increased, whereas the total chromatin length is very close to that in the root tip cells, it follows that in the cultured cells there must be significantly more DNA packed into each unit length of chromatin. From Table 1, it can be seen that the mean DNA content per micrometre of the CAPT genome is 46.6 arbitrary units, as compared with 38.3 arbitrary units for the root tip. Whether the DNA is in general more tightly packed in the CAPT genome, or whether there is simply a greater proportion of hetero-

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Table 1

Chromosome type	Root tip			Culture						
	L	SAT	S	1	2	3	4	5	6	7
Mean chromosome length (μm) (25 measurements of each) ± S.E.M.	8.45±0.22	6.32±0.16	3.72±0.09	8.01±0.2	7.74±0.19	6.53±0.17	5.73±0.15	4.23±0.08	4.28±0.11	2.72±0.08
Mean total genome length (μm)	36.98±0.47			39.24±0.98						
Mean C-band length (μm) per chromosome (8 to 10 chromosomes of each) ± S.E.M.	0.96±0.073	2.89±0.22	1.66±0.11	2.25±0.12	2.21±0.17	2.61±0.14	2.28±0.21	2.47±0.16	1.77±0.11	1.54±0.07
Mean C-band length (μm) per genome ± S.E.M.	11.02±0.40			15.13±0.98						
Mean fraction C-band material per genome	0.298			0.386						
Mean G 1 DNA content (100 measurements) ± S.E.M.	1,416±19.2			1,827±28.5						
Number of C-bands per genome	20+4 variable			26+1 variable						
Mean DNA content per micrometre of chromatin	38.3			46.6						

chromatin (which contains DNA in a tightly packed form) than in the root tip cells is not clear from this data alone.

Careful measurement of C-band regions in 10 chromosomes of each type has allowed construction of the idiogram in Fig. 1 *d*. Typical examples of each chromosome are depicted below the idiogram. There are 26 C-positive blocks consistently visible in the CAPT genome, whilst the centromeric block on chromosome 2 is not always seen. The mean fraction of C-band material (heterochromatin) per genome is 0.386 (see Table 1), which is 1.3 times greater than in the root tip. It was previously noted that the DNA content of the CAPT genome is also 1.3 times greater than in the root tip. Thus, it seems that this "extra" DNA in CAPT cells is entirely heterochromatic, and is concentrated into the C-band regions of the genome.

It should also be noted that there is a greater proportion of metacentric type chromosomes in the CAPT genome (5 out of 7 chromosomes) as compared with the normal diploid (4 out of 6 chromosomes).

4. Discussion

In this report, the patterns of C-banding along metaphase chromosomes of *Crepis capillaris* root tip are more clearly defined than in the two previous studies of this species (Schweizer 1973, Tanaka and Komatsu 1977). Additionally, both prior attempts failed to reveal as many C-banded regions as presently shown, although those bands which were reported by Tanaka and Komatsu have all been seen in the present study.

A single, consistent, C-banded karyotype has been seen in cells of the CAPT culture. Such dominance of a particular karyotype commonly occurs in plant cell cultures (Bayliss and Gould 1974, Singh 1975), presumably because cells containing a specific chromosome complement are better suited to survival in the specialized environment of a tissue culture system. In the case of this CAPT culture, the cell cycle in the dividing population is extremely short in duration, and the G_1 phase is virtually non-existent (Ashmore and Gould 1979). Thus, cell with the 7 chromosome complement observed in this cell line, may have dominated because of their capacity for such rapid cell cycle traverse. Certainly, it is known that changes in chromosome constitution can cause alterations in cell cycle timing (Krune and Wolf 1977). The proportion of heterochromatin in the chromosome complement may also affect cell division rate (Barlow 1973), so that the increased heterochromatin content of the CAPT cells may have affected the ability of these cells for survival in a cell population initially containing mixed karyotypes.

Recently, there has been some doubt expressed about the stem cell concept of animal tumours (Hashmi *et al.* 1974), since, in the original studies, the most reliable criterion of the stem cell was chromosome number. In the case of the CAPT culture, however, careful observation has revealed a single C-banded karyotype, but further investigation would be necessary to reveal

whether this apparent adherence to the stem line concept in the CAPT culture is a common characteristic of all plant tumours.

The hyperdiploid, 7 chromosome karyotype of the CAPT cell line deviates considerably from the normal diploid complement of *Crepis capillaris* (see Fig. 1). Bayliss (1980), has recently reviewed the available literature on chromosomal instability in plant tissue cultures. He comments that in nearly all of the 55 different plant species studied there is a deviation from the conventional karyotype of the cells of the intact plant. The abnormal karyotype observed in the CAPT culture is therefore consistent with these findings. A comparison of the CAPT chromosome complement with that of the normal diploid karyotype indicates that not only is there an extra chromosome present in the CAPT genome, but, additionally, all chromosomes differ in length from the original root tip chromosomes and have strikingly different C-band patterns, suggesting that numerous structural chromosomal rearrangements have occurred during the formation of the CAPT chromosome complement. Although chromosomal rearrangements are certainly observed in some plant tissue cultures (Bayliss 1980), and, particularly, in *Crepis capillaris* cell lines (Sacristán and Wendt-Gallitelli 1973), such extensive rearrangements have not previously been indicated. It may be that such major chromosomal change is associated with tumorous nature of the CAPT cell line, and that the integration of the Ti plasmid into the plant genome may possibly affect chromosomal stability. Alternatively, a similar degree of structural change may, in fact, also occur in other plant cell lines, but has not been revealed by previous studies, since banding techniques have not been used.

From a comparison of the C-band patterns of the CAPT genome with that of the root tip, it is possible to make certain suggestions about the derivation of the CAPT genome. Proposed schemes for the complete derivation of chromosomes 7, 6, and 3 are as follows:

(i) Chromosome 7 is most likely derived from the SAT chromosome as represented diagrammatically in Fig. 2. *a*. This involves breakage of the SAT chromosome between the centromere and the C-positive satellite blocks, *i.e.*, at the secondary constriction. Although no short arm is visible on chromosome 7, this may in fact be present, but difficult to detect, since the DNA at the site of a secondary constriction is highly despiralized. A considerable deletion of the long arm of chromosome SAT must also have occurred.

(ii) Chromosome 6 also appears to be derived from the SAT chromosome by a similar mechanism (see Fig. 2 *b*). However, in this case, there must be a smaller deletion of the long arm of SAT.

(iii) The slightly shorter arm of chromosome 3 of the CAPT genome may have been derived from the long arm of the SAT chromosome. Again, this would involve breakage at the secondary constriction, together with a deletion in the long arm. The longer arm of chromosome 3 may have arisen from chromosomes L and S, of the root tip complement, as set out in Fig. 2 *c*.

The site of the secondary constriction on the SAT chromosomes, where the nucleolus organizer region (NOR) is located, is a common breakage point in all three proposals. Such breakage at the NOR commonly occurs in other systems (JOHN and LEWIS 1968). Studies by SOKOLOV *et al.* (1974) on colchicine induced multinucleate cells of *Crepis capillaris* plants indicated that micronuclei are only capable of replication, and, hence, survival, if the NOR containing chromosome (SAT) is present. Thus, if the break at the secondary

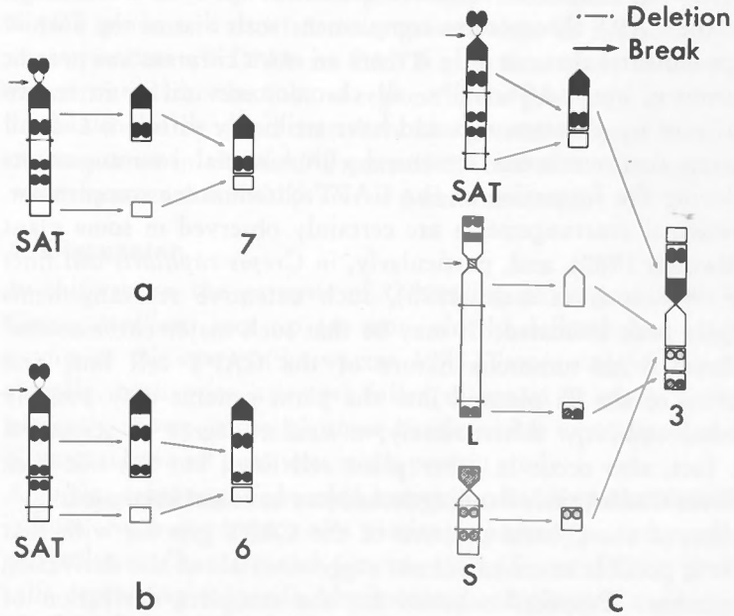

Fig. 2. *a* Scheme for the derivation of chromosome 7 of the CAPT genome from the SAT chromosome of the root tip. *b* Scheme for the derivation of chromosome 6 of the CAPT genome from the SAT chromosome of the root tip. *c* Scheme for the derivation of chromosome 3 of the CAPT genome, involving all 3 chromosome types (L, SAT, and S) of the root tip

constriction of the SAT chromosome in these proposed schemes is at the junction of the NOR with the chromosome arm, so that the NOR is lost as an acentric fragment, then it would have to be assumed that alterative NOR sites may have arisen. These could originate, *de novo*, through amplification of a dormant site. If, on the other hand, the breaks occur within the NOR itself, then the fractured NOR may still remain functional due to the repetitive nature of ribosomal DNA. Amplification of the sequences which remain is also possible. The application of techniques which cytologically stain NOR regions would obviously be of value to examine these possibilities.

It is also possible to propose explanations concerning the origin of parts of the

other four chromosomes of the CAPT genome. The long arm of chromosome 2 has most likely originated from the long arm of chromsome L, since both the telomeric and centromeric C-blocks of that chromosome are present. The spacing of the double C-band blocks on chromosomes 1 and 2 of the CAPT genome suggest that these may originate from the long arm of the SAT chromosome. Similar blocks also appear on chromosomes 4 and 5, with increased material in the lower block. It is also possible that these double blocks of C-positive material have arisen through differential replication of other regions of the genome, *e.g.*, the long arm of chromosome S.

Since more than two copies of chromosome SAT are implicated in the origin of the CAPT genome, there would have to be an initial evolution in the CAPT culture to a hyperdiploid karyotype, presumably to tetraploidy in the first instance. Polyploidization certainly occurs in other plant cell cultures (Bayliss 1980), and has also been reported in *Crepis capillaris* tissue cultures by many authors (Bayliss 1980). It is worth noting that polyploidy must arise *in vitro* in this species, since no polysomy has ever been observed in the whole plant (Brossard 1978). To obtain the abnormal aneuploid CAPT karyotype from the normal tetraploid complement, chromosomal loss, combined with rearrangements, must occur.

Aneuploidy may arise in tissue cultures by errors of division in polyploid cells. The large, vacuolated nature of plant cells in culture may be a cause of difficulties in the division process. Mitotic abnormalities (including lagging chromosomes at anaphase, chromosome bridges, multipolar spindles), are observed in plant tissue cultures (Bayliss 1973), and it is believed that such abnormalities are more readily tolerated by polyploid cells, since loss of chromatin leads to less genic imbalance than loss from a diploid cell. In *Crepis capillaris*, Sidorov and Sokolov (1963), have shown the appearance of up to 23% aneuploidy in plants following the induction of tetraploidy by colchicine treatment. Sacristán (1971), also observed progressive loss of chromosomes from a tetraploid clone of *Crepis capillaris* callus cells, to produce a hyperdiploid karyotype.

Chromosomal rearrangements are also known to occur more readily in polyploidized cells in culture than in diploid cells in both plant (Sacristán 1971) and animal systems (Hsu 1961, Halfer *et al.* 1980). All types of known chromosomal change (deletions, inversions, duplications, translocations) are detectable in polyploid *Drosophila* cell lines (Dolfini and Halfer 1978). Chromosomal change by translocation has previously been observed in *Crepis capillaris* (Sacristán and Wendt-Gallitelli 1973), and both translocations and inversions have been found in C-banded cells of *Vicia faba* callus (Papes *et al.* 1978). It is worth noting that an increase in heterochromatin content has been found to follow chromosomal rearrangement in cell cultures (Halfer *et al.* 1980), and such an increase is also known from studies in natural populations, *e.g.*, in some Australian frog species of the genus *Litoria*, in which

KING (1980) has proposed that "extra" heterochromatin has arisen both by amplification and by euchromatin transformation.

Thus, the proposed scheme for karyotype evolution, of tetraploidisation followed by segregation and rearrangement, has substantial support from similar studies in both plant and animal populations. Such a scheme was proposed by BAYLISS and GOULD (1974) to explain the origin of aneuploidy in *Acer pseudoplatanus* suspension cell cultures, and is also a common pathway of karyotype evolution in both normal and tumorous animal cells in culture

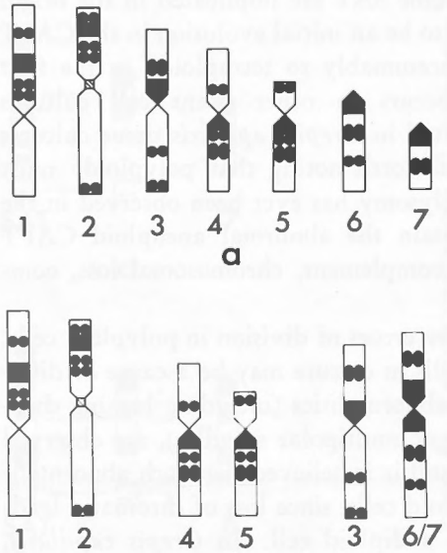

Fig. 3. *a* Idiogram of the C-banded karyotype of the CAPT cell line. *b* Idiogram of the 3 proposed "pairs" of chromosomes which may be derived from the CAPT karyotype by a simple fusion of chromosomes 6 and 7

(LEVAN and BIESELE 1958, HSU 1961, TERZI and HAWKINS 1975), and in *Drosophila* cell lines (DOLFINI and HALFER 1978).

By one simple change it is possible, on the other hand, to produce a 6 chromosome karyotype from the 7 chromosome karyotype observed in CAPT cells. This change involves fusion, at the centromeres, of chromosomes 6 and 7 of the CAPT genome. The resultant 6 chromosome set can then be arranged into 3 "pairs" with similarities of C-band pattern between the chromosomes of each pair (see Fig. 3 *b*). The production of this 6 chromosome set would not necessarily implicate polyploidisation as a preliminary step in the evolution of the CAPT karyotype. Such a system of karyotypic evolution, by-passing a polyploid step, would be unusual compared to other reports involving plant tissue culture, and might be associated with the tumorous state of the CAPT

culture. However, to obtain the 6 "pairs" of chromosomes from the original diploid set would not only require structural rearrangements of chromosomes in the form of inversions, but would also require a capacity for addition of both C-band positive and C-band negative material, and euchromatin transformation, *i.e.*, conversion of apparent euchromatic material to C-banded heterochromatin. Such complex karyotypic change is less likely to have occurred within a diploid genome, so that the preferred scheme for derivation of the CAPT karyotype from the normal diploid is that which involves initial polyploidy.

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PROTOPLASMA

Cell Cycle Analysis of Tumour-Derived Cultures of *Crepis capillaris*: A Kinetic Analogy With Proliferation of Animal Tumour Cells

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Summary

Analysis of the cell cycle by three methods has revealed unusual kinetics of proliferation in tumour derived suspensions of *Crepis capillaris*. The different methods of analysis yield different estimates of cycle phase durations, and such discrepancies have been explained in terms of low growth fractions with rapid total cycle traverse. Specifically, confidence in the estimation of G_2 duration by the fraction of labelled mitosis analysis, and comparison with shorter G_2 estimates obtained by the two other methods, suggests that cells drop out in G_1. However, cells which do not drop out of the proliferative compartment traverse G_1 extremely rapidly. Extremely short cell cycle durations in which the G_1 phase is virtually non-existent are uncharacteristic of plant cell suspension cultures, in which the G_1 phase has previously been shown to be extended as compared with meristematic root tip cells. A model has been proposed in which a central core of rapidly dividing cells continuously loses cells into a subpopulation of resting or G_0 cells with the G_1 DNA content. Similarities between plant and animal tumours with respect to cell growth and division are discussed.

Keywords: Crepis capillaris; Cell cycle; Tumour.

1. Introduction

A major feature of tumours is their capacity for essentially unrestrained or autonomous growth. Thus studies of the ways in which cells transformed to the neoplastic state differ from normal cells must consider alterations in the kinetics of cell growth and division. Such alterations, revealed by mitotic cycle analysis of mammalian tumours, include characteristic changes in cycle duration and growth fraction in the hyperplastic or neoplastic states

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(Bresciani 1968). A recent stochastic model of division control applied on a comparative basis to tumourous and non-tumourous animal cell cultures shows that tumour cells are less sensitive to withdrawal of serum than are normal cells (Shields and Smith 1977). An analogous property of plant tumour cells in cultures is their ability to continue growth in the absence of exogenously supplied hormones. However, as yet there are no reports concerning the cell cycle in plant tumours and even the normal division cycle has been studied mainly in non-plant systems (Mitchison 1971). The relatively few investigations into the plant cell cycle have utilised either organized meristematic populations (Howard and Pelc 1953, Wimber and Quastler 1963, van't Hof 1965) or tissue culture systems (Yeoman *et al.* 1966, Gould *et al.* 1974, Chu and Lark 1976). With one exception (Bayliss 1975) the tissue cultures used have all required the exogenous supply of at least one hormone.

In this report, using three methods of cell cycle analysis, the kinetics of cell proliferation have been described for a tumour-derived, hormone-independent suspension culture of *Crepis capillaris*. The application of more than one analytical approach has allowed cell cycle phase durations to be estimated and has revealed a rather complex kinetic situation which would not have been resolved by any one method. In this first step towards the understanding of the nature of cell proliferation in plant tumours, parallels have been found with the mammalian neoplastic cell cycle and, in particular, an unusually short G_1 period has been demonstrated.

2. Materials and Methods

2.1. Cell Line and Cell Cultures

The cell line, CAPT, used throughout this investigation is a derivative of a diploid ($2n = 6$), tumour-derived callus culture of *Crepis capillaris* kindly supplied by Dr. M.D. Sacristán. The original tumour was induced by *Agrobacterium tumefaciens* (Sacristán and Melchers 1970). A sensitive assay for octopine (Baldwin and Gresshoff 1978) failed to demonstrate the presence of this characteristic marker in the CAPT suspension cultures. However, the loss of such markers without loss of auxin autotrophy has been demonstrated previously (Marton *et al.* 1979).

Stock suspension cultures (60 ml) in 250 ml Erlenmeyer flasks were grown in the dark at 26 °C with rotary shaking (150 rpm with a ½″ throw). Transfers were made by inoculating 100 ml of 7 day old suspension into 50 ml of fresh B 5 medium with no added hormones (Gamborg and Eveleigh 1968). After 3 years under this suspension culture regime over 95% of dividing cells have a 7 chromosome aneuploid karyotype. Routine cytological monitoring has demonstrated the long-term stability of this aneuploid culture. Such stability precludes any errors in phase duration estimations due to progressive karyotype evolution. Tracheid elements were observed at a frequency of about 1%, indicating a very low leakage of cells into this differentiation pathway.

2.2. Estimation of Growth Parameters and Viability

Techniques for estimation of rates of cell number increase were as previously published (Gould 1977) with the following exceptions: aqueous chromium trioxide solution was used

at 10%; cell maceration was at 60 °C for 5 to 10 minutes, and cell lumps were dissociated by vigorous shaking for a similar period of time. Viability of cell suspensions was estimated by applications of Evans Blue, which enters only dead cells. The validity of this method has been checked by reciprocal staining with fluorescein diacetate, which causes live cells to fluoresce (WIDHOLM 1972).

2.3. Cell Cycle Analysis Methods

For the fraction of labelled mitosis analysis (FLM) cell suspensions were pulse labelled with tritiated thymidine (1×10^{-8} mol l^{-1} specific activity 5 Ci $mmol^{-1}$ [185 GBq $mmol^{-1}$] which gives an activity per ml of 5×10^{-2} µCi [1.85 kBq], Radiochemical Centre, Amersham) for 20 minutes followed by a cold thymidine chase (5×10^{-5} mol). A label-to-cold chase ratio of 1 : 5000 had previously been shown to be effective as a pulse labelling treatment for this culture. Cell cycle parameters were derived both manually, as described by MITCHISON (1971, p. 22), and also by computer, using the program of Dr. K. KOSCHEL.

For continuous labelling analyses, cell suspensions were repeatedly supplied with tritiated thymidine (1×10^{-8} mol l^{-1} every 6 hours). Scintillation counting had previously shown that a single application of tritiated thymidine at this concentration is totally utilized after 7 to 10 hours, whereas cells labelled every 6 hours incorporated tritium into their macromolecular fraction in an essentially linear fashion for at least 45 hours.

For both fraction of labelled mitoses and continuous labelling analyses, cell samples were fixed in 3 : 1 ethanol : acetic acid for at least 24 hours and autoradiographs of Feulgen stained cells were prepared using Kodak AR-10 stripping film. Autoradiographs were exposed for up to 14 days prior to development in Kodak D-19 and fixation in Ilford Hypam. Mitotic and labelling indices are means of counts of 2,000 nuclei, and the FLM parameter is calculated from observations of 100 mitotic figures for each data point.

2.4. Feulgen-DNA Densitometry (Microspectrophotometry)

Measurements of DNA content of individual nuclei in Feulgen-stained autoradiographs were performed on a Zeiss 02 photometer system at 570 nm. Integrated absorbance of specimens was automatically calculated by running the Apamos Modified program on a Digital PDP12 computer interfaced to the photometer. The ratio of G_1 to G_2 nuclei was estimated from DNA distributions of non-labelled interphase nuclei. Using this ratio and the relevant mitotic and labelling indices, cell cycle phase durations were determined by the method set out by CLEAVER (1967).

It was necessary to confirm that DNA synthesis was continuous in these cultures, otherwise interpretation of pulse and flash labelling methods is difficult. Thus, DNA contents of labelled nuclei in autoradiograph preparations were determined in pulse labelled populations by calculating the difference in absorbance at 570 nm and 670 nm. This technique is fully described elsewhere (GOULD 1979), and depends on the differential absorption of silver grains and the Feulgen-DNA dye complex. By this technique a study of rates of DNA synthesis as cells progress through S phase can be made if DNA precursor pools are small and/or constant in size.

Scintillation counts on cell samples removed from CAPT suspension cultures which were continuously supplied with tritiated thymidine revealed virtually no thymidine pools in these cells, and densitometric analysis indicated continuous DNA synthesis throughout the S phase.

3. Results

3.1. FLM Analyses and Growth Curves

Combined data from 5 separate investigations of cell number increase against time in suspension cultures (Fig. 1) indicate that there is no discernable lag

phase and that apparent exponential growth continues for approximately 72 hours after inoculation. The average cell doubling time during this exponential growth period is 37.6 hours. Two FLM analyses were performed on separate cultures, each between 24 and 66 hours after inoculation (Fig. 2). The durations of G_2, S, and the total cell cycle time have been determined, both by manually plotted FLM curves, and by computer analysis (see Materials and Methods) of raw FLM data (Tab. 1). Estimates of mitotic duration were obtained from scores of mitotic index, and the values for G_1 duration were calculated by difference. Although the interpeak distance in FLM plots is

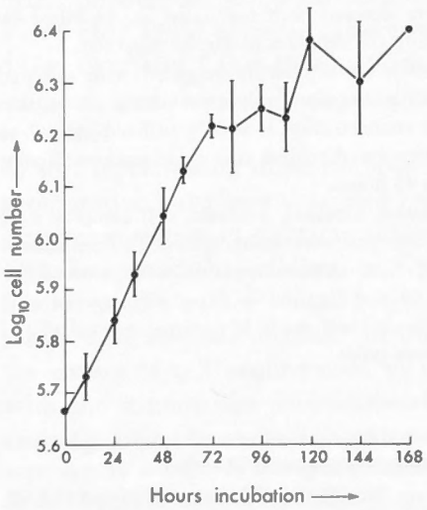
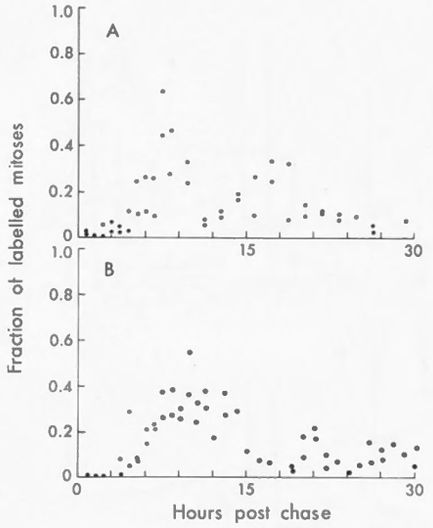


Fig. 1. Cell number increase curve of CAPT cell populations. The data are summations of counts on five separate cultures, each inoculated with cells grown at 26° for 7 days

considered a poor measure of total cycle time by proponents of recent probabalistic models of the cell cycle (SMITH and MARTIN 1973, GOULD 1977), such estimates of cycle time obtained from the FLM curves of cultures A and B indicate a disproportionately large discrepancy between estimates of total cycle time obtained by FLM analysis as compared with cell number increase data. This suggests that there may be a non-cycling fraction in the CAPT populations.

Both computer analysis and manual plotting of FLM data give closely comparable estimates of total cycle times but significantly different estimates for the subdivisions of the cycle. Very careful scoring methods, using randomized slides and multiple counts, failed to reduce the scatter of points on the FLM plots, which renders heuristic analysis (*i.e.*, curve fittings by eye) unreliable. The high degree of scatter on both the ascending and descending limbs of the first peaks in Fig. 2 may explain why the duration of S phase, in particular, is extended in the manual analysis (which takes account of all the data on the

broad peak) compared with the computer generated durations (which puts more emphasis on the top-most point, so that the half peak height level is elevated more than in the intuitive, manual method). Values for G_2 are in better agreement, as might be expected, because only one source of scatter (the ascending limb) is involved in the measurement of the G_2 transit time. It is noticeable that whereas the FLM curve for culture B shows a longer cell division time than for culture A, this lengthening is caused by increased durations of S, G_2 and mitosis, whilst the G_1 duration remains the same in both



Figs. 2 *A* and *B*. Fraction of labelled mitoses (FLM) curves for two CAPT cell populations

Table 1. *Accumulated Data of Estimated Phase Durations for Two Cultures of CAPT.* The data have been derived using both heuristic and computer analysis of FLM curves, and by applying Mak's combined autoradiography and densitometry method assuming all cells are cycling with total cycle times (Tc) estimated from relevant FLMs

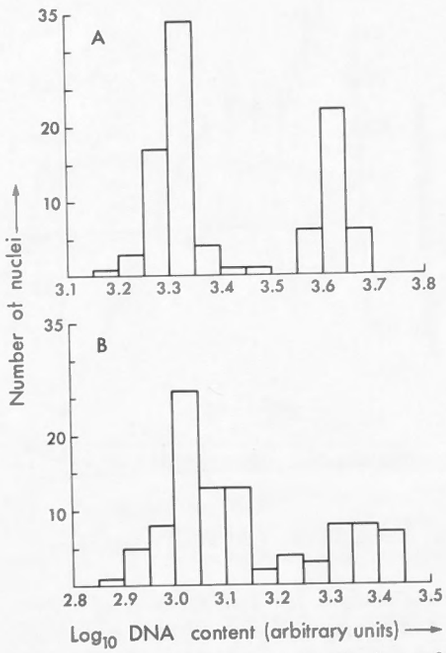
	Type of analysis	Phase durations (h) Tc ± S.E.M.	G_1	S ± S.E.M.	G_2	M
Culture A	FLM manual plot	10.4	0	3.6	6.2	0.6
	FLM computer analysis	9.13 ± 0.2	3.17	1.62 ± 0.1	3.94 ± 0.5	0.4
	Mak's method	9.13 ± 0.2	4.1	0.53	4.3	0.25
Culture B	FLM manual plot	14.4	0	6	7.3	1.01
	FLM computer analysis	13.01 ± 0.3	3.28	1.95 ± 0.1	7.38 ± 0.1	0.4
	Mak's method	13.01 ± 0.3	7.2	1.38	4.32	0.37

cultures (Tab. 1). Such elongation of cell cycle time may be explained by slight alterations in culture conditions. However, previous studies with non-tumourous suspension cultures have indicated that extension of cell division

time in different cell cultures of the same cell lines, is due to variation in G_1 duration alone, with G_2 and S durations remaining constant (GOULD *et al.* 1974).

3.2. *Combined Densitometry (Microspectrophotometry) and Autoradiography*

These analyses were carried out on samples withdrawn from cultures A and B (referred to in the FLM analyses) 20 minutes after addition of tritiated thymidine. In Feulgen-stained autoradiographic preparations of these samples all



Figs. 3 *A* and *B*. Frequency distributions of values for Feulgen-DNA contents of unlabelled interphase nuclei in rapidly dividing CAPT cultures (24 hours after inoculation). Each distribution represents the staining density of 100 nuclei, which showed no grain (*i.e.*, G_1 and G_2 nuclei) in autoradiographs of cells exposed to tritiated thymidine for 20 minutes. Differences in scale range arise from variation in overall Feulgen staining intensity in different staining runs

labelled cells are in S phase, unlabelled cells are either in G_1 or G_2, depending on their DNA content, and mitotic cells are distinguished cytologically (MAK 1965). Fig. 3 presents Feulgen densitometry data obtained from the zero hour samples of the A and B FLM curves. The ratios of G_1 to G_2 cells (0.54 : 0.39 for culture A; 0.64 : 0.26 for culture B), labelling and mitotic indices (0.057, 0.019 for culture A; 0.096, 0.019 for culture B), and cell cycle times derived from the relevant FLM curves were combined to calculate cycle phase-durations (Materials and Methods). The interphase durations determined by this meth-

od differ significantly from those obtained by FLM analysis (Tab. 1). With the exception of the G_2 estimate for culture A, densitometric analysis gives lower values for S and G_2 than the FLM method. Conversely, the FLM estimates of G_1 are much shorter than values assigned by densitometry. Mak's method of cell cycle analysis gives valid estimates of cycle phase durations only in situations where very high proportions of cells are engaged in cell cycle

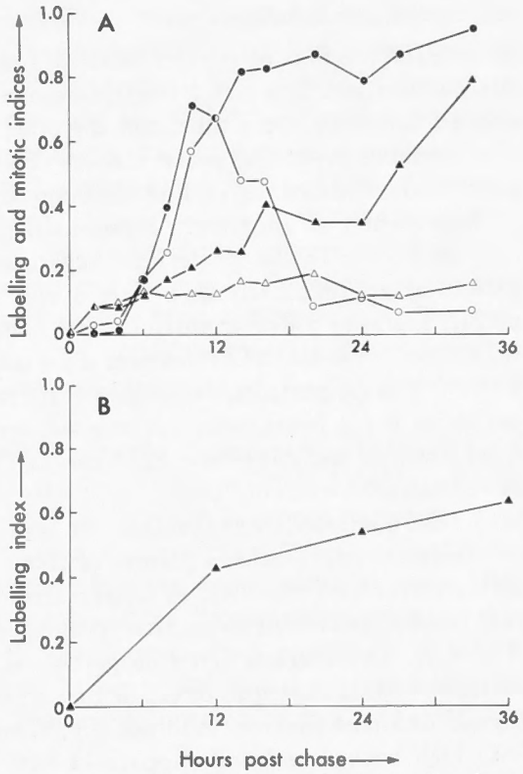


Fig. 4 *A*. Accumulation of labelled mitoses and total labelled cells in CAPT cell cultures after (i) single 6 hours dose of tritiated thymidine (1×10^{-8} mol l^{-1}) [—△— labelling index; — ○ — fraction of labelled mitoses], and (ii) multiple doses of tritiated thymidine (1×10^{-8} mol l^{-1} every 6 hours) [—▲— labelling index; — ● — fraction of labelled mitoses]. Each data point represents counts on 2,000 nuclei for labelling index, and 100 mitotic figures for determination of the fraction of labelled mitoses

Fig. 4 *B*. Theoretical accumulation of labelled cells with multiple dose continuously labelled cultures, according to the model proposed in the text

traverse. In contrast, FLM analyses depend on counts of labelled mitotic figures and so non-cycling cells cannot bias estimation of phase transit times obtained from the first peak. Thus the disagreements between the results obtained by the two methods can be explained in terms of low growth fractions in the CAPT suspension cultures. Since estimates of the length of G_1 are great-

est by the densitometric method, while the same analysis gives values for S and G_2 duration which were shorter than values from FLM analysis, it is logical to assume that non-cycling cells are arrested in G_1. A subpopulation suffering G_1 arrest would be expected to bias calculations of phase durations such that G_1 is overestimated, by Mak's method whilst other phases are underestimated.

3.3. Continuous Labelling

Counts for fraction of mitoses labelled and labelling indices were made on two cultures, one in which a single dose of tritiated thymidine was given for 6 hours starting at zero time, and the other in which the culture was dosed with tritiated thymidine every 6 hours (Fig. 4 *A*). This second labelling regime made tritiated thymidine continuously available to the cells over a 45 hour period of apparently exponential growth in the cultures (Materials and Methods). In the single dose experiment, the labelling index shows a plateau at approximately 0.16 after 6 hours, whilst the fraction of labelled mitoses begins to rise at approximately 4 hours and peaks at 0.76 (higher than for the pulse labelled FLM analyses since the cohort of labelled cells is much wider). The G_2 parameter estimated from both single and multiple dose experiments is 6.5 hours, which is in good agreement with the value obtained from heuristic analysis of the FLM curves. Thus, this value for G_2 duration can be accepted with confidence.

With label continuously available in the multiple dose experiment, the fraction of labelled mitoses reaches a plateau of about 0.8 after 15 hours and rises only slowly thereafter (Fig. 4 *A*). A small fraction (approximately 5%) of mitoses still remain unlabelled even after prolonged supply of tritiated thymidine (Fig. 4 *A*). This suggests that a proportion of cells in the cultures may become "arrested" either in mitosis or G_2, or that a number of cells are defective in the thymidine kinase enzyme. Additionally, human error in counting or problems with high background and slippage of film in autoradiograph preparations may account for part of the apparent unlabelled fraction. At 33 hours, 80% of total cells show label and after continuous labelling for the presumptive cell cycle time of 9 to 14 hours, labelling index is only 0.3. Results of the continuous labelling experiments and a comparison of the time courses for fraction of mitoses labelled, and total labelled cells, again suggests the existence of a non-cycling sub-population in the suspension.

4. Discussion

No single analytical technique, applied in isolation, gives a satisfactory description of the dynamics of the cell cycle (Painter *et al.* 1964, Mendelsohn and Takahashi 1971, Gould *et al.* 1974). In the tumour-derived *Crepis capillaris* suspension cultures even analysis by three different methods does

not yield an immediately comprehensible model of the kinetics of cell proliferation. These difficulties in interpretation arise from the presence of sizeable non-cycling subfractions in the CAPT cell populations which adversely affect the accuracy of the continuous labelling and densitometric methods. The resolution of the FLM analysis is reduced by intrinsic features of the CAPT cell cycle, namely, the relative durations of S, G_2 and M.

The FLM analysis is most successful in situations where S phase is long relative to G_2 and mitosis. In this situation S phase cells traverse rapidly into mitosis allowing little asynchrony to develop in the labelled cohort and the relatively narrow mitotic "window" is saturated with labelled cells for an easily measured period (*i.e.*, the breadth of the well defined peak is the duration of S phase). These conditions apply particularly to the mammalian cell cycle (QUASTLER and SHERMAN 1959, BASERGA and WIEBEL 1969). However, in CAPT suspensions the duration of G_2 appears to be long in comparison with S phase (Tab. 1). Thus, during the long G_2 stochastic processes may develop asynchrony within the labelled cohort and the mitotic "window" is unlikely to become saturated by the relatively narrow band of S phase cells. The flattened indistinct nature of the FLM curves in this analysis (Fig. 2) can therefore be understood in terms of the temporal structure of the S-G_2-mitosis sequence.

The FLM peak after a single dose of tritiated thymidine not followed by a cold chase, reaches a value of 76% at 10 hours (Fig. 4 *A*), and the duration of G_2 as estimated from this curve is 6.5 hours, which is in good agreement with the pulse chased FLM curves. Therefore the majority (80%) of cells traverse G_2 in 6.5 hours and also the highest labelling index obtained, even after 33 hours of continuous labelling is 80%. This discounts the possibility that the very small second peaks in FLM represents a sub population of CAPT cells having a G_2 duration of about 14 hours and thus the distance between the two peaks can be taken as an approximate measure of total cell cycle time (see Materials and Methods).

Extrapolation from the data on continuous labelling yields an estimate of total cycle duration of 46 hours if all cells are assumed to be cycling (method of CLEAVER 1967). Substituting this estimate, in place of the FLM-derived value for total cycle duration, phase durations have been recalculated from the densitometry data (Mak's method) and are presented in Table 2. In this recalculation both the G_1 and G_2 parameters are significantly longer (20.7 hours, 25.2 hours and 21.6 hours, 15.2 hours, respectively) than those transit times as measured from the FLM curves (Tab. 1). However, the directly measured time taken for cells to traverse G_2 is close to 6.5 hours in 4 separate analyses (Figs. 2 and 4 *A*), and thus the assumption that all cells are cycling is again called into question. Additionally, population doubling time as calculated from cell number increase curves is about 37 hours, and therefore the estimate of 46 hours for total cell cycle time derived from the continuous

labelling data must be discounted. Taking the total duration of the cycle as between 9 and 14 hours (FLM analyses), the continuous labelling data (Fig. 4 *A*) can only be interpreted in terms of a low growth fraction in the CAPT cultures.

If all cells in the population are assumed to be cycling and cell division time is taken as 12 hours (FLM analyses), predictions of increase in cell number with time can be made. Comparison between predicted and observed cell number increases, and estimates of the fractions of cells which must therefore leave the cell cycle at each stage of the growth cycle, are presented in Table 3.

Table 2. *Recalculation of Phase Durations for Two Cultures Assuming (i) All Cells Cycle and Total Cycle Time (Tc) is 46 Hours (From Continuous Labelling Data), and (ii) G_1 Cells Are Non-Cycling and Tc is as Calculated by FLM.*
Note that the second assumption gives estimates which agree most closely with FLM derived durations, especially for G_2 (Tab. 1)

	Type of analysis	Phase durations				
		Tc	G_1	S	G_2	M
Culture A	Mak's method, assuming all cells cycle	46	20.7	2.7	21.6	1.2
	Mak's method, assuming all G_1 cells are non-cycling, and taking Tc from FLM analysis	9.13	0	0.85	7.94	0.53
Culture B	Mak's method, assuming all cells cycle	46	25.2	4.8	15.2	1.3
	Mak's method, assuming all G_1 cells are non-cycling, and taking Tc from FLM analysis	13.1	0	2.6	9.75	0.98

Using this approach it appears that 0.77 of cells inoculated do not enter the division cycle, and, subsequently between 0.2 and 0.4 of the dividing population exit from the cycle per division. Applying this model of a continual drop out of cells from the division cycle, a plot has been made of the expected values for fractions of cells labelled during continuous labelling of CAPT suspension cultures between 24 and 72 hours of culture (Fig. 4 *B*). This predicted plot fits quite closely with the experimental plot obtained (Fig. 4 *A*). Similarly, application of this "continual drop-out" model predicts that 0.6 of the population will be in the non-dividing compartment after 24 hours of culture (Tab. 3), which is when the densitometric analysis were carried out. This fraction is very close to the experimentally determined proportion of the population which is in G_1 at this time (Fig. 3). Since it has already been argued that non-cycling cells are arrested in G_1 rather than in any other cell cycle phase [see Results, Combined Densitometry (Microspectrophotometry) and Autoradiography], it is suggested that the majority of G_1 cells in these cultures is in a resting state. This agrees with data obtained from the FLM curves which indicate that the G_1 phase is either completely absent (heuristic analysis), or at least of very short duration (computer analysis), in cycling cells. Recalcula-

tion of all phase transit times by applying Mak's method, and excluding all G_1 cells from these estimations, gives values for the lengths of G_2, S and mitosis which are in much closer agreement with the values obtained from the FLM analyses (Tab. 2).

Thus, the "continual drop-out" model which accommodates all of the data and considerations presented above, has the following characteristics:

(i) Cycling cells have either a very short, or a non-existent G_1 phase.

(ii) In both these tumourous cultures and in root meristems of *Crepis capillaris* the durations of the S, G_2, and mitotic phases are similar (LANGRIDGE *et al.* 1970, GENERALOVA 1969, KUROIWA and TANAKA 1969, ABRAHAM *et al.* 1968, VAN T HOF 1965) with the possible exception of S phase, which may be shorter in the cultures. (For CAPT cultures $G_1 = 0 \rightarrow 2$ hours, $S = 2 \rightarrow 4$ hours, $G_2 = 6.5$ hours, $M = 1$ hour.)

Table 3. *Cell Number Increases for Successive Time Intervals Modelled on a 12 Hours Division Cycle (FLM Data).*
Cycling and non-cycling fractions are calculated from observed cell number increase (Fig. 1) compared with expected cell number increase if all cells divide

Time (h) interval	Cell No. increase (cell $ml^{-1} \times 10^{-5}$)	Fraction of cells assumed to leave division cycle	Fraction of cells in cycling compartment after each interval	Fraction of cells in non-dividing compartment after each interval
$0 \rightarrow 12$	$4.525 \rightarrow 5.546$	0.774	0.248	0.752
$12 \rightarrow 24$	$5.546 \rightarrow 6.918$	0.327	0.398	0.602
$24 \rightarrow 36$	$6.918 \rightarrow 8.811$	0.315	0.427	0.573
$36 \rightarrow 48$	$8.811 \rightarrow 10.97$	0.423	0.397	0.603
$48 \rightarrow 60$	$10.97 \rightarrow 13.87$	0.339	0.412	0.587
$60 \rightarrow 72$	$13.87 \rightarrow 16.60$	0.511	0.339	0.661

(iii) During proliferation in culture a relatively constant proportion of the cycling populations arrest in G_1 (0.3 to 0.4 per cycle).

This last concept of cell arrest in G_1 has parallels with both the Q cell (quiescent cell) proposal of CHU and LARK (1976) for soy bean cell cultures, and the G_0 non-cycling compartment proposed by LAJTHA (1963) and EPIFANOVA and TERSKIKH (1969). It may be that G_1 arrested cells in the CAPT cultures have lost the ability to initiate DNA synthesis, and such loss of competence could be related to the total number of cycles a given cell has undergone in its history (HAYFLICK and MOORHEAD 1961). Alternatively, the clumps of cells which occur in CAPT populations may represent meristematic nodules, and the G_1 arrested cells in these cultures may have lost the ability to cycle through separation from such parental clumps. Certainly, according to the "continual drop-out" model, a large fraction of the inoculated cells in CAPT cultures fail to enter the division cycle. However, recently proposed non-deterministic

models of cell division control do not require the existence of a non-cycling sub-fraction of the population to explain G_1 "arrested" cells. Rather, such cells are considered still division-competent and have not irrevocably left the cell cycle (Shields and Smith 1977). In terms of the transition probability model (Smith and Martin 1973), cells in the CAPT suspensions have a probability of initiating a new round of DNA synthesis and division of either close to zero (those cells which become arrested in G_1), or close to one (those cells which traverse G_1 very rapidly).

Previous reports have shown that the plant cell cycle is very much extended in suspension culture as compared with meristematic root tip cells of the same species (Bayliss 1975) and, in particular, it is the G_1 phase which is increased in length in hormone-requiring suspension cultures (Gould 1977). However, this analysis of tumourous CAPT suspension cultures indicates a total cycle duration close to that in root tips of the same species and the G_1 transit time, rather than being extended, is of extremely short duration. Thus the possibility arises that although exogenously supplied auxin is essential for growth in non-tumourous plant cell cultures, such growth is achieved only at the expense of an extended G_1 period. Bayliss (1975) has reported a shortened cell cycle time (32.8 hours) during embryogenesis in auxin-free suspension cultures of *Daucus carota*, as compared with the normal non-embryonic cultures to which 2,4-D was supplied (cycle time = 50.9 hours). In particular, the G_1 phase was most reduced in the auxin-free culture. Parallels with the CAPT suspension culture, therefore, suggest a link between auxin autotrophy and a shortened cell cycle time, in which the G_1 phase is specifically most reduced in duration. This raises the possibility that the exogenously supplied auxin (2,4-D) is directly inhibiting the rapid transit of cells from G_1 into the DNA synthesis period.

In CAPT cultures the growth fraction is less than one because a proportion of the cycling population arrests in G_1. In cycling cells G_1 is very short or absent, and the total cycle time of 9–14 hours is uncharacteristically short for a plant cell suspension culture. These unusual kinetics also feature in animal tumour cell populations, where low growth fractions (not necessarily smaller than in the corresponding normal populations—Gavosto and Pileri 1971) commonly occur (Mendelsohn 1962, Baserga 1965, Bresciani 1968), as do shortened cycle times (Reiskin and Mendelsohn 1964, Dormer *et al.* 1964, Iverson *et al.* 1962, Bresciani 1965), and absence of G_1 in the cycling cells (Baserga 1963, Lala and Patt 1966). Likewise, dividing CAPT cells that are autotrophic for auxin, with their rapid transit through G_1, are similar to virally-transformed animal cells, which are far less sensitive to reductions in serum levels in terms of proliferation than are their untransformed counterparts. This suggests that a general feature of tumourous cells (both plant and animal) may be the ability to traverse the G_1 phase rapidly and somewhat independently of culture conditions.

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Cell cycle related changes in the quantity of TMV virions bound to protoplasts of *Nicotiana sylvestris*

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Summary

Attachment of virions of tobacco mosaic virus to protoplasts isolated from dividing, suspension cultured cells of Nicotiana sylvestris was estimated using quantitative autoradiography of individual protoplasts. Additionally, the position of each protoplast in the cell cycle was assessed by Feulgen microspectrophotometry. At pH 5.6, after preincubation with 4 g. mL^{-1}. poly-L-ornithine, protoplasts in the G_1 and G_2 phases bound more virions than protoplasts in the S-phase. The possibility that such differential binding was caused by cyclical variation in the net charge on the protoplast membrane has been investigated. It was found that S-phase protoplasts of N. sylvestris can be separated from protoplasts of other cycle stages by partition in aqueous, two-phase, immiscible polymer systems, presumably because they differ in charge. Also, electrophoretic studies suggest that G_1 phase protoplasts bear higher surface charge than some non-G_1 protoplasts.

1. Introduction

The interaction of plant virus virions with leaf protoplasts *in vitro* has been intensively studied, primarily as a model of the infection process (TAKEBE and OTSUKI 1974, BURGESS et al. 1973), and quantitative investigations using either radioactively labelled virions (ZHURALEV et al. 1975), fluorescent antibody (OTSUKI and TAKEBE 1969) or electron microscopy (COUTTS et al. 1972) have been aimed at optimising virion binding, uptake and replication. However, for certain animal viruses, it has been shown that infection can depend on the host cell's position in the cell cycle (EREMENKO et al. 1972, YANAGI et al. 1977), with the S-phase of synchronized cell populations being most susceptible (LAWRENCE 1971, FURUKAWA 1979) and thus virus attachment and infection may be interpreted as a probe for physiologial changes related to cell cycle progression. Unfortunately, parallel studies on cell cycle related variations in susceptibility to plant viruses have not been reported, possibly because most *in vitro* studies have been done with protoplasts from mature leaves, most cells of which are in the G_1 phase and thus furnish protoplast populations which are undifferentiated in terms of cell cycle stage.

Recent reports have indicated that the initial binding of virions to plant protoplasts is determined by electrostatic charge interactions dictated by the iso-electric point of the virion, the pH of the protoplast culture medium, and the negative potential of the protoplast membrane (OKUNO and FURUSAWA 1978, NAGATA and MELCHERS 1978). Preliminary autoradiographic observations concerning

cycle related variation in binding of virions by plant protoplasts (GOULD 1979) may be interpreted as due to changes in surface charge of protoplasts at different stages of the cell cycle. Such variation in virion binding is probably not significant in terms of the infection process per se, because of the exclusively G_1 condition of the cells of most mature leaves and also because relatively high levels of polycation are needed to elicit this cell cycle-related effect.

2. Materials and Methods

2.1 Cells media and protoplast preparation

A callus culture of Nicotiana sylvestris was kindly supplied by Dr P. Dix, who derived the tissue from haploid material. A cell line (NS-1) from this callus was maintained as a suspension culture in the medium RM 1964 (LINSMAIER and SKOOG 1965) with 2,4-D at 0.4 mg L^{-1}, kinetin at 0.03 mg L^{-1}, sucrose at 40 g L^{-1} and casimino acids at 1 g L^{-1}. Every 7 days 10 ml of the cell suspension were transferred into 50 ml of fresh medium and incubated at 26°C at a shaking rate of 150 excursions per minute in a Gallenkamp orbital shaking incubator. After 36 months of this transfer regime, all cells in mitosis showed 48 chromosomes (i.e. they appeared to be tetraploid).

Protoplasts were prepared from cultures 2 days after transfer by centrifuging 10 ml of cell suspension and resuspending the cells in protoplast washing buffer (P.W.B.: 2 mM NaH_2PO_4, 3 mM $CaCl_2$, 11% sorbitol; pH 5.6). Washed cells were finally resuspended in 10 ml of enzyme mix (2% Driselase, 2% pectinase, 1% hemicellulase, in P.W.B.) and shaken gently at 50 r.p.m. and 26°C in a Petri dish. Protoplast release was usually complete after 3 to 4 h incubation. Protoplasts were separated from whole cells and wall debris by gently washing the enzyme/cell mixture through 63 m Nytal gauze. After a final resuspension in 3 ml P.W.B. better than 98% of protoplasts were viable, as assessed by Evans Blue exclusion and fluorescein diacetate staining (WIDHOLM 1972), and at this stage, Calcofluor White staining did not reveal the presence of cell wall material.

2.2 Viruses

The virions of four tobamoviruses were used in this work. The type strain (U1) of tobacco mosaic virus (TMV) and the U2 strain (T_2MV) were obtained from Dr K. Helms (CSIRO, Canberra). They were grown in Nicotiana clevelandii Gray and virions (^{3}H-TMV and ^{3}H-T_2MV) labelled with ^{3}H uridine were prepared as described by GIBBS and MacDONALD (1974). Virions of two other tobamovirus strains (TOM-1 and TAB-1) were kindly supplied already labelled with ^{3}H-uridine by Dr L. Pelcher (Prairie Regional Laboratory, Saskatchewan, Canada). Also, preliminary experiments were done using tobacco necrosis satellite virus donated, with its helper virus by Dr R.I.B. Franki (Waite Institute, Adelaide). This satellite virus (SV) was grown in N. clevelandii and its virions were labelled with iodine -125 by the method described by STANLEY and HASLAM (1971).

2.3 Autoradiography

Protoplasts exposed to tritiated virions were fixed in a mixture of 9 volumes ethanol, 3 volumes glacial acetic acid, 4 volumes water, with sorbitol at a final concentration of 11%. After 1 h protoplasts were sedimented by brief centrifugation and resuspended in 3:1 ethanol-glacial acetic acid and left overnight. Protoplasts were then rehydrated, Feulgen stained, and resuspended in 25% acetic acid. Drops of this suspension were placed on subbed slides and allowed to air dry. Slides were left overnight, then immersed in distilled water prior to application of Kodak A.R. 10 stripping film. Autoradiograph slides were

then stored in dry, light-tight boxes for exposure periods of 4 to 10 days at 10°C. Slides were developed for 6 minutes in Kodak D-19 developer and fixed in Ilford Hypam. Microscopic examination was done directly under oil without application of coverslips to the autoradiographs.

2.4 Microspectrophotometry and automated grain counting

All measurements were made with a Zeiss photometer (interfaced to a PDP-12 computer using the APAMOS MODIFIED programme) by the silver grain removal method described by GOULD (1979). In this method two measurements are made on each Feulgen-stained protoplast in an autoradiograph preparation. After the first measurement of the absorbance at 570 nm of individual Feulgen stained cells and autoradiographic silver grains associated with them, the slide is treated with Farmers Reducer (a ferricyanide/ thiosulphate reagent which removes silver grains). Then individual protoplasts are relocated and measured again using the APAMOS "Find and Adjust" memory option, and the difference between first and second measurements assesses the absorption drop due to silver grain removal (i.e. the number of virions bound). The second measurement also gives an estimate of the position of the protoplast in the cell cycle assessed from its nuclear DNA content (GOULD 1979). Thus each protoplast can be assigned a cell cycle position and a virion binding ability, and if sufficient protoplasts are analysed, a picture of how such binding ability varies through the cell cycle can be synthesized.

2.4 Protoplast electrophoresis

The cell electrophoresis apparatus was similar to that described by NAGATA and MELCHERS (1978). However in our apparatus certain difficulties were encountered with temperature fluctuations and electro-osmotic effects and measurements of electrophoretic velocities were not considered reliable enough for the calculation of absolute millivolt potentials on individual protoplasts. Protoplasts used for electrophoresis were isolated either from exponentially dividing or from stationary phase cell populations and some measurements were made in the presence of poly-L-ornithine. Measurements were made at or very close to the stationary points of the electrophoresis cell. Corrections for the slight drift caused by electro-osmosis were achieved by measuring protoplast velocity relative to the movement of uncharged silica particles included in the electrophoresis buffer. Results are expressed as velocities in arbitrary units (eyepiece micrometer division per unit time).

2.5 Two-phase separation

This method relies on the differential partition of protoplasts between two immiscible phases, in this case dextran and polyethylene glycol. The partition of protoplasts depends on the relative affinities of the membrane surface for the polymers present in the two phases and the protoplasts' absorption at the interface. The addition of appropriate salts to the system can produce an electrostatic potential difference between the two phases. Such charge differentiation increases the separation of cell types which differ in surface charge

(REITHERMAN et al. 1973). In this study the very efficient separation which can be achieved by a counter-current distribution method (ALBERTSSON 1970) was exploited, and the phase system most suitable was found to consist of 6.3% dextran 500, 6.3% PEG 4000, 10 mM KPO_4 buffer at pH 7.8, 10 mM NaCl. When protoplasts had been partitioned in the counter-current distribution apparatus, the samples were centrifuged, washed in PWB to free them of polymer and then fixed as described for Feulgen staining and autoradiography.

3. Results

3.1 Virus binding experiments

Preliminary experiments with radio-iodinated virions of tobacco necrosis satellite virus (^{125}I-SV) confirmed that electrostatic considerations could account for the initial interaction between these virions and Nicotiana sylvestris protoplasts. SV was used because its virions have an isoelectric point of 7.0 (KASSANIS 1970) which is high compared with the virions of other plant viruses, most of which have isoelectric points around 4 to 5. ^{125}I-SV was added to protoplasts in buffers over the pH range 5.6 to 7.5 and incubated at 26°C for 10 minutes. The quantity of ^{125}I-SV bound to the protoplasts was measured by scintillation counting of thoroughly washed protoplasts. Protoplasts in pH 5.6 buffer bound 3 times as much ^{125}I-SV as did protoplasts in pH 7.0 and 7.5 buffer. We conclude that where the protoplast buffer pH is close to the isoelectric point of the virions, electrostatic interaction between them and the negatively charged protoplast membrane is diminished.

In contrast with the ^{125}I-SV results, insignificant amounts of radioactivity were bound to N. sylvestris protoplasts when they were incubated with tritiated virions of all tobamoviruses tested, over the same range of buffer pH. The isoelectric points of all tobamovirus virions are close to 3.5 (ZAITLIN 1970) and this should preclude virion binding at pH values in the range 5.6 to 7.5 because both virions and protoplast membrane will be negatively charged. Unfortunately we could not easily produce adequately

tritiated tobacco necrosis satellite virus and ^{125}I-SV is not suitable for the quantitative autoradiographic method (GOULD 1979). Therefore tritiated tobacco mosaic viruses were used in this study, even though the electrostatic interactions between TMV virions and protoplasts require the presence of polycations for efficient binding. OKUNO and FURUSAWA (1978) have demonstrated the role of polycations, such as poly-L-ornithine, in virus infection of barley protoplasts, and so an experiment was done to define the optimum level of polyornithine for efficient binding of ^{3}H-TMV whilst maintaining protoplast viability. It was found that the viability of protoplasts decreased rapidly at polyornithine concentrations above 4 µg ml^{-1}. However it is likely that exposures longer than a few minutes to polyornithine at 4 µg ml^{-1} would also prove toxic to protoplasts. In these experiments it was only necessary to demonstrate viability in the short term, as protoplasts were fixed after 3 minutes of incubation. Autoradiography showed that ^{3}H-TMV was very efficiently bound at polyornithine concentrations above 1 µg ml^{-1}, with progressively increasing autoradiographic grain densities up to 100 µg ml^{-1} polyornithine.

Protoplasts isolated from 2 day old suspension cultures of N. sylvestris, and resuspended in 3 mls of P.W.B. (pH 5.6) to give 2 x 10^{5} protoplasts ml^{-1}, were incubated with 4 µg ml^{-1} polyornithine for 60 seconds. Then 0.05 ml (1.8 x 10^{5} c.p.m.) of a suspension of ^{3}H-TMV (approximately 1 mg ml^{-1}) was added to the protoplasts and after 2 minutes the sample was fixed. Feulgen stained

autoradiographic preparations of such protoplasts demonstrated that only about 70% of the protoplast population had bound virions (Fig. 1a & b). It was also evident that the presence or absence of radioactivity (i.e. of virion attachment) was correlated with nuclear morphology and size. This observation suggested a correlation between the ability to bind TMV and the position of the protoplast in the cell cycle. Fig. 2 shows seven protoplasts, four of which have small, condensed nuclei and associated silver grains (i.e. they have bound ^{3}H-TMV virions), and three of which have diffuse nuclei and show no sign of radioactivity.

The absorptions at 570 nm of 200 Feulgen stained protoplasts, randomly selected in an autoradiograph preparation, were measured with the Zeiss microspectrophotometer system before and after removal of silver grains, the co-ordinates of each protoplast were stored by the computer. Results (Fig. 4) demonstrate that although protoplasts in the G_1 and G_2 stages of the cell cycle varied greatly in their ability to bind virions, S-phase protoplasts bound much less virus than protoplasts in other stages of the cell cycle. Protoplasts in mitosis are rarely identified in such experiments, perhaps because they are fragile and lost during isolation, or because the high osmotic pressure of the protoplasts' culture conditions causes clumping of chromosomes thus obscuring mitotic figures. The few protoplasts seen in mitosis have more associated silver grains (i.e. have bound more virions) than S-phase protoplasts (Fig. 3). This apparent reduction in the proportion of mitotic figures is the only distortion in cell

cycle distribution of N. sylvestris protoplast populations as compared to the exponentially dividing cell suspensions from which they are isolated (GOULD unpublished data). Several other similar analyses, using virions of TMV and T2MV, and the two strains TOM-1 and TAB-1 supplied by Dr Pelcher, invariably demonstrated that S-phase protoplasts of N. sylvestris bound fewer virions than G_1 and G_2 protoplasts. However distribution of protoplasts in different parts of the cell cycle ensures that only about 10% of protoplasts are in S-phase, and so measurements of 200 randomly selected protoplasts gave only about 20 protoplasts in the low-binding S-phase. Therefore another analysis was done, comparing the cell cycle distribution of protoplasts with heavy grain densities with that of lightly labelled protoplasts. Results of these analyses are given in Table 1. In these experiments polyornithine was used at 4 $\mu g\ ml^{-1}$ as previously, but 0.2 ml of the ^3H-TMV suspension was mixed with 3 mls of protoplast suspension to give virion saturation. In this experiment all protoplasts became labelled. Protoplasts were classed as either heavily labelled or lightly labelled before removal of grain with Farmers Reducer. After grain removal protoplasts were relocated and absorbances were read at 570 nm. From Table 1 it can be seen that the lightly labelled protoplast sub-populations contained up to 40% S-phase protoplasts, whereas the heavily labelled sub-populations had less than 1% S-phase protoplasts (actually only 1 S-phase in 150 protoplasts measured in the first analysis).

In all of the virion attachment experiments, duplicate

samples were pre-stained with Evans Blue before Feulgen staining, in order to identify dead protoplast, a method kindly suggested by Dr Lyndsey Withers. In all experiments less than 4% of protoplasts were dead and there was no preferential binding of virions by such dead protoplasts.

3.2 Protoplast electrophoresis

For reasons outlined previously, absolute millivolt potentials were not calculated for protoplasts in this study. Electrophoretic mobilities of about 50 protoplasts obtained from a stationary phase culture, were compared with mobilities of protoplasts isolated from an exponentially dividing culture. Feulgen microspectrophotometry of stationary phase protoplasts demonstrated that over 95% of the population was in the G_1 phase. Histograms of the distributions of protoplast velocities are displayed in Fig. 5. The upper histogram (Fig. 5A) derives from stationary phase (G_1) protoplasts, the lower (Fig. 5B) from exponential (cycling) protoplasts. It appears that a significant proportion of protoplasts derived from exponential cultures migrates more slowly than stationary phase protoplasts, under the standard conditions of electrophoresis. Electrophoresis of protoplasts in the presence of polyornithine at 4 $\mu g\ ml^{-1}$ abolished or reversed protoplast migration but very variable results were obtained.

3.3 Two phase separation

Protoplasts isolated from dividing cultures were partitioned by a counter current distribution method as

described. It is very significant that changes in NaCl concentration (which affect the potential difference between the 2 polymer phases) greatly altered the partition of protoplasts. Results of Feulgen microspectrophotometry of two fractions obtained by such partition are presented in Fig. 6. Fig. 6A shows the bimodal distribution of Feulgen-DNA content of protoplasts obtained by bulking contents of tubes 12, 13 and 14 of a 30-tube distribution experiment. Fig. 6B shows the intermediate single peak distribution of Feulgen-DNA contents derived from protoplasts distributed in tubes 20, 21, 22 and 23 of the same experiment. The histogram in Fig. 6A is characteristic of a population containing all phases of the cell cycle. The Fig. 6B histogram, because of its intermediate position in terms of DNA content, would appear to represent mainly S-phase protoplasts. This result suggests that there is a characteristic differentiation in terms of surface properties of S-phase protoplasts as compared to protoplasts in G_1 and G_2. It must be re-emphasised that the influence of altered NaCl concentrations on partition suggests that separation of protoplasts is due to charge differences.

4. Discussion

Our experiments indicate that protoplasts of Nicotiana sylvestris which are in the S-phase of the DNA replication-partition cycle are less susceptible to polyornithine mediated binding of TMV virions than are protoplasts in other phases of the cell cycle. This is not the first time that virus/host-cell interactions have been

shown to be cell-cycle dependent and the importance of the S-phase has been specifically mentioned in this context by other authors (LAWRENCE 1971, FURUKAWA 1979). However, all previous work on the relation between cell division phase and the mechanisms of virus infection and replication has been with animal cells (GROYON and KNIAZEFF 1967, EREMENKO et al. 1972, ST. JEOR and HUTT 1977, YANAGI et al. 1977) so the experiments with plant protoplasts reported here provide a plant-kingdom parallel with such previous investigations.

Several parameters which correlate with the dynamic structure of the cell membrane, including microviscosity (de LAAT et al. 1977) and cellular electrophoretic mobility (MAYHEW 1966) have been shown to be cell cycle related. If, as has been previously reported, the initial stage of virus attachment is mediated by simple electrostatic interaction (OKUNO and FURUSAWA 1978, NAGATA and MELCHERS 1980) it might be postulated that the differences in TMV virion attachment, reported here, reflect cycle-related variation in the charge on the protoplast membrane. Such an interpretation is most easily understood if the poly-ornithine used in the ^{3}H-TMV experiments is thought of as the primary probe for the gross electronegative charge on individual protoplasts. Under the experimental conditions used (i.e. pH 5.6) the protoplasts bound no TMV virions in the absence of polyornithine, presumably because both protoplasts and virions were negatively charged. When polyornithine is added to a protoplast suspension the number of polycationic molecules bound to the protoplasts should be related to the magnitude of the negative charge on the

membranes, that is, protoplasts with highest net negative charge bind most polyornithine. Virion attachment will then depend on the positive charges displayed by the polyornithine molecules bound to each protoplast. Our experiments, if interpreted using such a scheme of charge interaction, suggest that S-phase protoplasts carry less net negative charge (and so bind less polyornithine and therefore less ^{3}H-TMV) than protoplasts of other cycle phases.

There is some previously published support for the idea of electrostatic charge variation on the membrane during the cell cycle. For instance MAYHEW (1966) reported that synchronized, human osteogenic sarcoma cells in culture, showed greatest electrophoretic mobility during mitosis and attributed this to an increase in surface charge density. The virus binding results taken in isolation do not constitute strong evidence for a decrease in electrostatic charge on the protoplast membrane during DNA synthesis because of the complex nature of the binding process (i.e. polyornithine is the mediator of successful binding). We were unable to obtain tritiated polyornithine, which would have allowed direct autoradiographic quantification of the amount of polycation which binds to protoplasts of different cycle phase. However the two phase separation technique lends strong support for a charge difference between S-phase protoplasts and G_1 or G_2 protoplasts (Fig. 6), because alterations in NaCl concentration caused redistribution of protoplasts between the two phases (although hydrophobic

interactions can also play a part in separations obtained by this method). In this study the two-phase technique did not establish whether S-phase protoplasts had elevated or depressed surface charge as compared with G_1 and G_2 protoplasts.

The limited data obtained from protoplast electrophoresis experiments are sufficient to indicate that protoplast populations obtained from dividing (i.e. cycling) cell suspensions contain an "electrophoretically slow" subpopulation as compared to the predominantly G_1 protoplast populations obtained from stationary phase cell suspensions (Fig. 5). Thus, S-phase protoplasts, which bind less TMV in the presence of polyornithine, and partition in a charge related manner in a two-phase separation system, may represent the slower fraction observed in the electrophoresis experiments.

No other authors have reported data relating changes in surface charge to cell cycle stage of plant protoplasts. HALIM and PEARCE (1980) have recently measured electrophoretic mobilities of protoplasts obtained from suspension cultures of Parthenocissus tricuspidata, but as the cultures used were 4 to 6 weeks old it is unlikely that the cells were cycling, and mobility measurements were derived for whole populations rather than single protoplasts. However the approach used by these authors (HALIM and PEARCE 1980) demonstrates the importance of an understanding of the surface properties of plant protoplasts, especially when considering manipulations concerning protoplast fusion or the uptake of viruses or foreign genetic material by protoplasts.

In summary, results obtained in this study with protoplasts of N. sylvestris suggest that there is a cyclical variation in the composition of the protoplast membrane, such that the net negative charge falls during G_1, is lowest during S-phase and rises again during G_2. This charge variation apparently dictates that S-phase protoplasts bind less TMV virions in the presence of poly-L-ornithine. The variation in the amount of ^3H-TMV bound to G_1 and G_2 protoplasts (Fig. 4) is consistent with the idea that their negative charge decreases during G_1 and increases during G_2, because the technique used to assign protoplasts to particular phases of the cell cycle does not distinguish between early or late fractions of G_1 or G_2 (GOULD 1979). From a virological standpoint it is important to note that infectivity may not necessarily be directly related to the number of virions bound. Also we do not know if protoplasts of other plant species show a cyclical variation in net negative charge so it is possible that the phenomenon reported here is peculiar to protoplasts from dividing cultures of N. sylvestris. Experiments with protoplasts derived from a tumour line of Crepis capillaris (ASHMORE, unpublished data) also suggest cycle related changes in surface charge, but the pattern of variation is not the same as proposed here. However, studies with cultured animal cells have shown how the topography and density of anions on membrane surfaces can vary with the state of the cell (BORYSENKO and WOODS 1979) especially with regard to teratogenic transformation.

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TABLE 1. Proportion of cells in different stages of the cell cycle that became lightly labelled† or heavily labelled when exposed to ^{3}H-TMV

		Stage of cell cycle			Number of cells measured
		G1	S	G2	(n)
Expt. 1	Heavy label	0.27#	>0.01	0.72	150
	Light label	0.33	0.36	0.30	99
Expt. 2	Heavy label	0.38	0	0.62	120
	Light label	0.39	0.40	0.21	100

† Lightly labelled cells had <10 grains per protoplast; heavily labelled >40 grains.

Proportion of cells in each stage

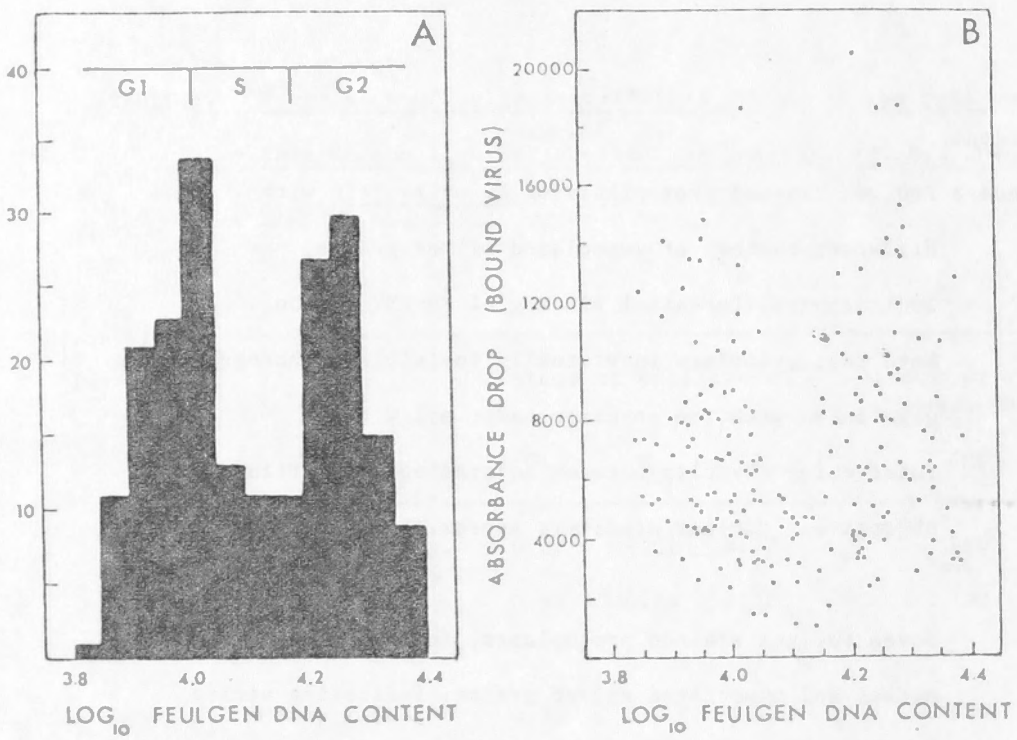
Figure Legends

Fig. 1A and B Feulgen stained protoplasts of *N. sylvestris* with different numbers of associated silver grains, indicating differential binding of ^{3}H-TMV virions. Note that cytoplasm is virtually invisible. Photographs were taken with the specimen under oil with no intervening coverlip between autoradiographic film and objective. Nuclear diameters approx. 10-20 m.

Fig. 2 Seven Fuelgen stained protoplasts, four with condensed nuclei and associated silver grains, indicating virion binding, the other three with diffuse nuclei and no apparent virion attachment.

Fig. 3 One of the few protoplasts found in mitosis, showing some associated silver grains.

photographs missing



4A Frequency distribution histogram of Feulgen-DNA values of protoplasts isolated from 2 day old N. sylvestris suspension culture.

B Scatter diagram showing the Fuelgen-DNA content of each protoplast plotted against the reduction in absorbance at 570 nm due to the removal of silver grains over the protoplast. The absorbance drop (indicating the number of virions bond) is quoted in arbitrary units obtained from the microspectrophotometer.

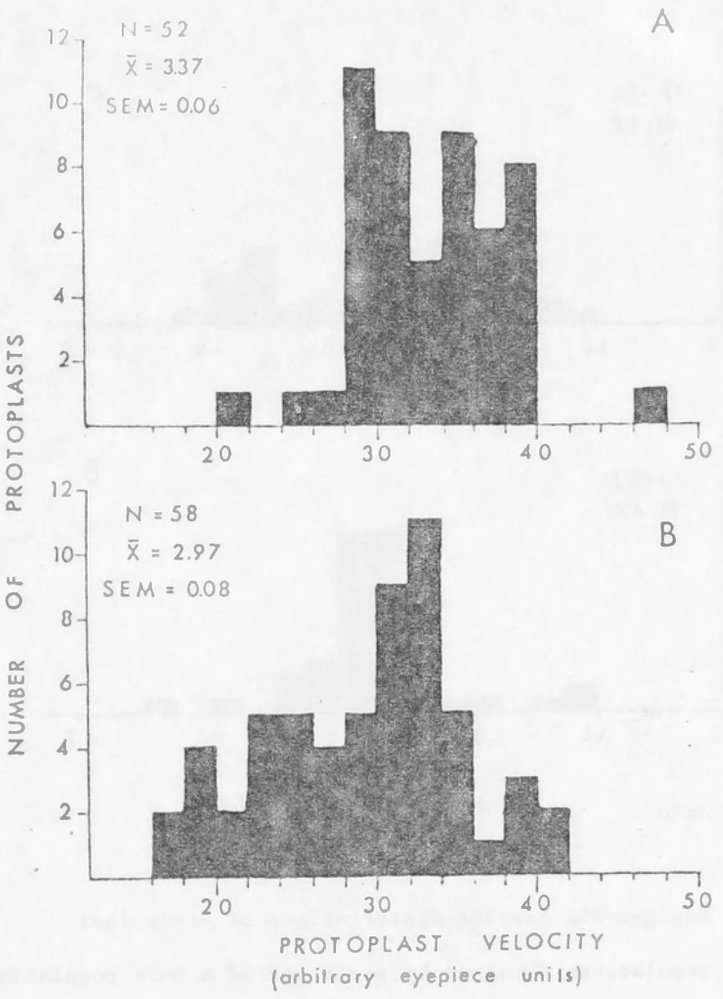


Fig. 5 Frequency distributions of electrophoretic mobilities of protoplasts isolated from suspension cultures of N. sylvestris.

A Distribution of mobilities (expressed as protoplast velocities in arbitrary units) of 52 protoplasts isolated from a stationary phase, 10 day old, suspension culture.

B Distribution of mobilities of 58 protoplasts isolated from an exponentially dividing, 2 day old, suspension culture.

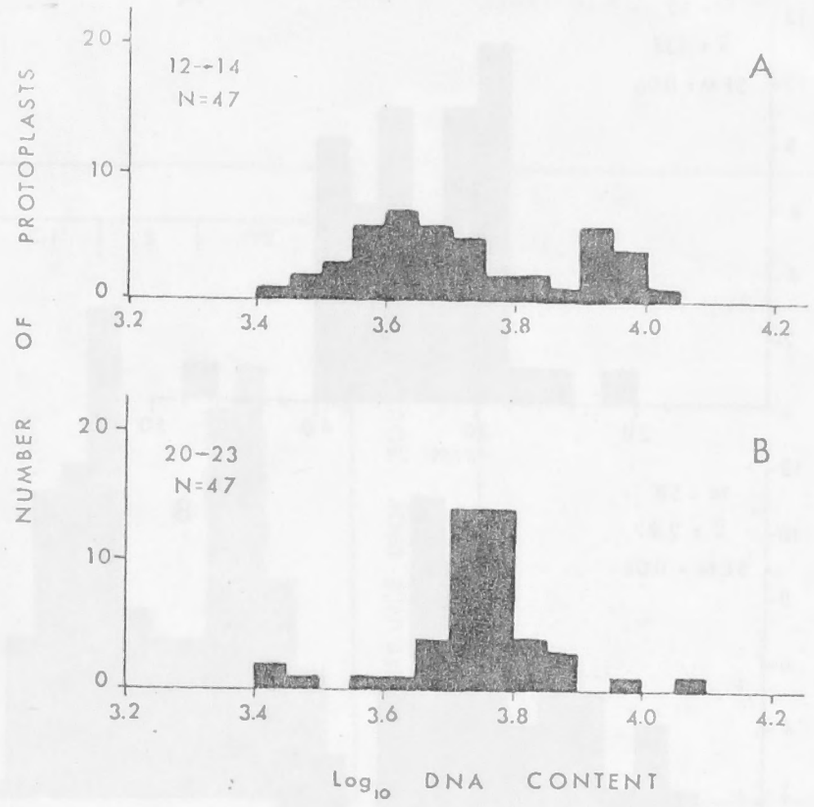


Fig. 6 Feulgen-DNA content distributions of protoplast populations obtained by partition of a bulk population of protoplasts of N. sylvestris in an aqueous two phase polymer system containing 10 mM NaCl. The original bulk population was derived from a dividing culture, contained representatives of all cell cycle phases, and was partitioned by counter current distribution in 30 tubes.

A A biphasic distribution with peaks corresponding to G_1 and G_2 DNA contents, obtained from tubes 12 to 14.

B A single peak distribution with the majority of DNA contents intermediate to the G_1 and G_2 peaks of Fig. 6A. This "S-phase" population was obtained from tubes 20 to 23.